The significance and clinical associations of autoantibodies in systemic sclerosis patients and their relatives

Harvey, Georgina Ruth

Award date:
1998

Awarding institution:
University of Bath

THE SIGNIFICANCE AND CLINICAL ASSOCIATIONS OF AUTOANTIBODIES IN SYSTEMIC SCLEROSIS PATIENTS AND THEIR RELATIVES

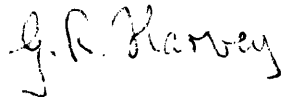
Submitted by

Georgina Ruth Harvey B.Sc. (Hons)

for the Degree of Doctor of Philosophy

at the University of Bath

1998



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To Nick and little Hannah

ABSTRACT

Systemic sclerosis (SSc) is a connective tissue disease characterized by skin thickening and autoantibodies (AAbs). Generally, only one or two of a limited number of SSc-specific AAbs are detected, and their identification is useful for diagnosis and prognosis in individual cases. Certain environmental agents have been implicated in the aetiology of SSc, and immunogenetic associations of some AAbs have been established.

The relative influence of genetic and environmental factors on the production of anti-nuclear antibodies (ANAs) and anti-nucleolar antibodies (ANoAs) was addressed in 62 SSc patients, and in their 215 first-degree relatives and 24 spouses. By indirect immunofluorescence (IF), a significantly increased incidence of ANoAs was detected in the blood-relatives of SSc patients, but not in their spouses, when compared with normals. These results suggest a heritable factor may cause the nucleolus to become a focus of the immune response in SSc patients and their relatives. When analyzed by radio-immunoprecipitation (IP) techniques, ANoAs of defined specificity were shown to be SSc-specific. However, some family members had strong IF-ANoAs, and also precipitated autoantigens of unknown identity by IP. In one family, the spouse and three sisters of a proband all had ANA-IF, and each was found to precipitate a strong band of 115-kDa. Antigen depletion and affinity purification experiments suggested that the spouse and one sister recognized the same protein, while the other two sisters recognized a different autoantigen. These results strongly suggested that, due to genetic differences, the same environmental insult had triggered disease in the proband, while, in the remaining family members, the sequelae had been limited to a non-pathological autoimmune response.

The expression of AAbs and their clinical associations were then examined by IP in a further cohort of 148 SSc patients. It was confirmed that three major subsets of SSc patients are each characterized by a particular form of disease expression, and by the presence of a particular, mutually exclusive SSc-specific autoantibody, *viz.* anti-centromere, anti-topoisomerase I (anti-topo I), and anti-RNA polymerase III (anti-RNAP III) AAb groups. Anti-RNAP III antibodies were often accompanied by antibodies recognizing RNAP I, and sometimes also RNAP II. Despite their different clinical associations, anti-topo I sera also often had anti-RNAP II antibodies: however, in many cases, only the phosphorylated form of RNAP II appeared to be recognized by anti-topo I sera, a finding confirmed by affinity purification studies. These data suggested that anti-RNAP II AAbs can be produced by two alternative immune response pathways, possibly involving different initiating stimuli.

Together, these results are consistent with the current model of aetiopathogenesis in which autoantibodies, while not being directly involved in disease pathogenesis, are, nonetheless, extremely reliable reporters of disease-specific pathological phenomena.

ACKNOWLEDGEMENTS

This work was generously supported by grants from the Raynaud's and Scleroderma Association, and the Arthritis and Rheumatism Council, U.K.

Clinical details were provided by members of the U.K. Systemic Sclerosis Study Group, and by clinicians from the Royal National Hospital for Rheumatic Diseases, Bath, U.K., including Professor P.J.Maddison, Dr N.J.McHugh and Dr Y.Patel. Special thanks to Sister Susan Butts for help with reviewing case notes, and for explaining medical terminology. I am grateful to Professor U.-F. Haustein, University of Leipzig, Germany, to Professor J.Craft, Yale University School of Medicine, U.S.A., and to Dr Sylviae Muller, Institut de Biologie Moleculaire et Cellulaire, Strasbourg, France, for supplying some of the sera used in the present studies. Thanks also to Dr I.Targoff, Department of Medicine, Oklahoma University Sciences Centre, Oklahoma, U.S.A. for confirming the specificity of the anti-PL-7 serum.

I would like to thank all at the Bath Institute for Rheumatic Diseases for the friendly and supportive atmosphere which I have enjoyed during my time there. My particular thanks go to Dr Jean Whyte for being a stimulating colleague, for her excellent technical demonstrations, and for making available her topoisomerase I functional assay results. Special thanks to Mr J. Dixey for his endless patience, support and instructive discussions; to Dr Susan Walsh for advice; to Mrs Maureen Jacobs for her kindness; to Ms Alison Rands for help and encouragement; to Dr Cally Jefferies for her concern; to Mrs Juliet Dunphy for positive thinking, support and technical troubleshooting, and to my supervisor, Dr N.J. McHugh for his great patience and understanding.

Thanks also to my sister Belinda for her support and encouragement. Finally, thanks to my partner Nick for help with references, patience, tolerance and support, and to our little daughter Hannah, just for being there.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 6: DISCUSSION

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AAb	autoantibody
ACA	anti-centromere antibody
ACE	angiotensin-converting enzyme
ADCC	antibody-dependent cell cytotoxicity
AMP	ammonium persulphate
ANA	anti-nuclear antibody
ANoA	anti-nucleolar antibody
APC	antigen-presenting cell
ARA	American Rheumatism Association
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bFGF	basic fibroblast growth factor
C	complement protein fragment; constant region of Ig
CENP	centromere protein
CNBr	cyanogen bromide
CTD	connective tissue disease
CTE	calf thymus extract
CTRD	carboxy terminal repeat domain
DABCO	1,4-diazabicyclo-[2,2,2]-octane(triethylenediamine)
DC	dendritic cell
dc-SSc	diffuse cutaneous systemic sclerosis
dFCS	dialysed foetal calf serum
DLCO	carbon monoxide diffusing capacity of the lungs
Dm	dermatomyositis
DMF	dimethyl formamide
DMP	dimethyl pimedilate
DNA-PK	DNA-dependent protein kinase
EC	endothelial cell
F_{ab}	antibody-binding fragment/domain of immunoglobulin
Fas-L	fas ligand
FB	fibroblast
F_c	crystalline fragment/domain of immunoglobulin
FCS	foetal calf serum
FITC	fluorocein isothiocyanate
GVHD	graft versus host disease
HLA	human lymphocyte antigen
hnRNP	heterogeneous nuclear RNP
IB	immunoblotting

ICAM	intercellular adhesion molecule
IF	indirect immunofluorescence
IFN	interferon
IL	interleukin
IL-2R	interleukin-2 receptor
IP	immunoprecipitation
IPP	protein immunoprecipitation buffer
J	joining region of Ig
K cell	killer cell
lc-SSc	limited cutaneous systemic sclerosis
LAK	lymphokine-activated killer cell
LFA	lymphocyte function-associated antigen
mAb	monoclonal antibody
MCTD	mixed connective tissue disease
mFas	membrane-bound fas ligand
MHC	major histocompatibility complex
MIP	modified (low-salt) IPP buffer
MNC	mononuclear cell
MS	multiple sclerosis
NBT	nitro blue tetrazolium (2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4,4'-diphenylene]-ditetrazolium chloride)
NET-2	RNA immunoprecipitation buffer
NK cell	natural killer cell
NOR	nucleolus organizer region
NP-40	nonidet P-40
PA	pernicious anaemia
PADPRP	poly(ADP-ribose) polymerase
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCA	phenol-chloroform-isoamyl alcohol
PDGF	platelet-derived growth factor
Pm	polymyositis
PVDF	polyvinylidene fluoride
RA	rheumatoid arthritis
RLE	Ro/La extract
RNAP	RNA polymerase
RNHRD	Royal National Hospital for Rheumatic Diseases
ROS	reactive oxygen species

RP	Raynaud's phenomenon
RPMI	Rosewell Park Memorial Institute 1640 medium
rRNP	ribosomal RNP
RR	relative risk
RTE	rabbit thymus extract
SDS	sodium dodecyl sulphate (lauryl sulphate)
sFas	soluble Fas ligand
Si-SSc	silica-associated SSc
SLE	systemic lupus erythematosus
snRNP	small nuclear RNP
SS	Sjogren's syndrome
SSc	systemic sclerosis
TBE	tris-borate-EDTA
TBS	tris-buffered saline
Tc cell	cytotoxic T lymphocyte
TC	tissue culture
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	transforming growth factor
Th cell	helper T lymphocyte
TIE	topoisomerase I extract
TNF	tumour necrosis factor
topo I	topoisomerase I
Tris.Cl	tris[hydroxymethyl]aminomethane hydrochloride
Trizma base	tris[hydroxymethyl]aminomethane
Ts cell	suppressor T lymphocyte
Tsk	tight-skin mouse
TSS	total skin score
UsnRNP	uridine-rich small nuclear RNP
UCTD	undifferentiated connective tissue disease
V	variable region of Ig
VCAM	vascular cell adhesion molecule
VLA	very late antigen

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 CLINICAL FEATURES

Systemic sclerosis (SSc) is a connective tissue disease (CTD) characterized by inflammatory microvascular lesions, increased deposition of extracellular matrix, and the presence of a limited number of disease-specific autoantibodies in patient sera. While its most obvious clinical manifestation is a thickening of the skin known as scleroderma, the increased mortality of SSc is associated with the involvement of internal organ systems (for reviews see (24,84,229,265,304,362)). The pathogenesis of SSc is not fully understood, and its aetiology is unknown.

Disease severity varies widely across the SSc spectrum, and is reflected by the degree and spread of cutaneous involvement. However, two main subtypes of SSc are recognized (198): in the limited form skin thickening is confined to the extremities and any serious visceral complications are late to develop, while in patients with diffuse disease, tethered, fibrotic skin extends over much of the body and there is early, often fatal, involvement of internal organs.

The reported prevalence of SSc is 100-200 cases per million, apparently increasing over the last few decades (326). However, this trend may be explained by changes in clinical criteria, modern study methods, and the inclusion of previously misdiagnosed and subclinical cases. The disease affects mainly women (F:M sex ratio >3:1), with onset typically at age 30-50. There are no particular geographical or racial proclivities, although some immunogenetic associations are emerging, and there may be a tendency in the families of some patients towards CTDs in general. There is convincing evidence that certain environmental agents are involved in the development of at least some clinical variants of scleroderma.

At present there is no cure for the disease itself: however, by reducing damage to internal organs, certain drug regimes have significantly improved mortality rates. While treatments remain inadequate and sometimes dangerous, accuracy of diagnosis and prognosis in this highly variable disease is an important aspect of patient care (23). An early designation of disease subtype serves to define the natural history of the disease, which is of primary concern to both patient and physician. The patient can be informed of the likely course, severity and time-scale of the disease, while the physician is alert to possible visceral complications. The costs and benefits of each available treatment can then be balanced appropriately.

Diagnostic criteria, disease subtypes and related disorders

Skin changes are the mainstay of diagnosis and classification in SSc. Sclerodermatous skin is tight, thick and characterized by non-pitting induration (218). According to the established criteria of the American Rheumatism Association (ARA), a diagnosis of generalized scleroderma, i.e. SSc, is reached on the basis of sclerodermatous skin extending beyond, and usually including, the digits (218). This is termed proximal scleroderma. However, less obvious symptoms such as sclerodactyly (digital

scleroderma), digital pitting scars, and certain characteristic symptoms of organ dysfunction may also lead to a diagnosis of SSc (for detailed clinical criteria see (218)).

The majority (60%) of SSc patients have limited cutaneous SSc (lc-SSc), where scleroderma affects only the hands, feet, face and forearms. The minority with the more severe diffuse cutaneous disease (dc-SSc) tend also to have upper limb and truncal skin involvement. The division of SSc into subtypes based on cutaneous distribution has been validated by consideration of the contrasting patterns of visceral involvement and serological findings in limited and diffuse disease. These, and other subsets of SSc, may represent aetiologically distinct disease processes or, alternatively, may reflect differences in patient vulnerabilities (86,198). The natural histories of the limited and diffuse forms of SSc will be described presently.

Localized forms of scleroderma are characterized by isolated patches of inflamed, fibrotic skin: vasculature is normal, and there are no visceral complications. Morphea appears as circumscribed plaques of affected skin: these may be isolated (limited morphea) or widespread (generalized morphea). Linear scleroderma occurs as bands of fibrotic skin, and is most often found in children, for whom growth abnormalities or disfigurement may be the consequence (229).

A rare variant of SSc, where the characteristic vascular changes and visceral involvement occur in the absence of cutaneous fibrosis, is known as SSc *sine* scleroderma.

As well as satisfying criteria for SSc, some patients exhibit features of other CTDs, and such cases are termed overlap syndromes. These include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), polymyositis/dermatomyositis (Pm/Dm), and Sjogren's syndrome (SS) overlaps. Undifferentiated connective tissue disease (UCTD) describes those patients whose few early symptoms are common to more than one CTD. The existence of mixed connective tissue disease (MCTD) as a distinct disease entity is controversial (319,22): the disease is defined by the presence of high titre anti-U1 RNP antibodies, and was originally described as a mild disorder, rarely affecting the lungs or kidneys. Some clinicians believe that such patients actually have UCTD, and it has been shown that many so-called MCTD patients subsequently satisfy the criteria for SSc, SLE, myositis or RA.

A number of environmentally induced scleroderma-like syndromes have been reported, such as vinyl chloride disease caused by the aliphatic hydrocarbon vinyl chloride, toxic-oil syndrome caused by aniline-contaminated rapeseed oil, eosinophilia-myalgia syndrome resulting from L-tryptophan supplements, and silica-associated SSc (Si-SSc), which can result from occupational exposure to silica dust. The aetiology of SSc is discussed further in Sections 1.5 and 1.6.

Vascular abnormalities and Raynaud's phenomenon

Structural and functional abnormalities of the microvasculature are present early in the course of SSc, most notably in the fingers. Alterations to capillary architecture are best

observed by widefield microscopic examination of nailfold capillary loops. Areas of enlargement and distortion are apparent, sometimes to the point of capillary destruction. Large conglomerations of dilated capillaries and venules at the skin surface, known as telangiectases, may be directly visible, and usually occur on the fingers and face.

At autopsy, digital arteries appear narrowed, sometimes being blocked by fibrotic deposits. Skin biopsy samples also show evidence of arteriolar fibrosis. Small arteries are affected first, with a concentric pattern of intimal proliferation (40). Such microvascular obliteration can ultimately affect visceral vasculature.

Further evidence of vascular abnormality in early SSc includes a high incidence of Raynaud's phenomenon (RP) (for review (16)), an episodic digital ischaemia provoked by cold or emotion. In a typical attack, vasospasm causes the fingers (and/or other extremities) to become numb and remarkably pallid; this is followed by pain, venostasis, and cyanosis due to ischaemia; finally, the attack ends with the return of bloodflow, marked by a hyperaemic recovery phase during which the fingers appear reddened. In SSc patients with severe RP, chronic ischaemia may result in digital ulcerations and occasionally gangrene. There is evidence that the visceral vasculature of SSc patients is also affected by Raynaud's-like abnormalities and vasospasms, with consequent ischaemia and organ damage. Isolated (primary) RP is very common in the general population (~5-10%): however, its almost universal presence in SSc (~98%), being the first symptom in over 70% of patients, highlights its importance when considering populations at risk of developing the disease: identifying the small proportion of individuals presenting with RP who will go on to develop SSc or some other CTD is a key aim of clinical research and practice. The pathogenesis of RP and SSc may indeed be related, with severe RP being a *formes frustes* of SSc (i.e. an extremely mild variant on the SSc disease spectrum, or an incompletely expressed form of the disease).

Cutaneous involvement

While the pace and extent of skin changes in SSc vary widely, three main phases are recognized: oedema, induration and atrophy. The oedematous phase is characterized by stiff, swollen hands and feet, and the appearance of RP. The phase of induration follows, during which the dermis of fingers and other affected areas becomes thick, shiny, taut and fibrotic; the epidermis thins, the production of sweat and sebum is reduced, loss of hair and skin creases is apparent, and there may be areas of hyperpigmentation. Tethering of skin to underlying tendons and joints often causes contractures and friction rubs, and may severely affect hand function and overall mobility. Skin biopsy reveals an abnormal accumulation of collagen fibres in the dermis. Facial skin is often affected, producing a characteristically expressionless face, with thin lips and small, tightened mouth. After some years the atrophic phase begins, and dermal skin may soften: however, epidermal atrophy, scarring and contractures remain.

Large conglomerations of dilated capillaries and venules at the skin surface, known as telangiectases, may be directly visible, and usually occur on the fingers and face.

At autopsy, digital arteries appear narrowed, sometimes being blocked by fibrotic deposits. Skin biopsy samples also show evidence of arteriolar fibrosis. Small arteries are affected first, with a concentric pattern of intimal proliferation (40). Such microvascular obliteration can ultimately affect visceral vasculature.

Further evidence of vascular abnormality in early SSc includes a high incidence of Raynaud's phenomenon (RP) (for review (16)), an episodic digital ischaemia provoked by cold or emotion. In a typical attack, vasospasm causes the fingers (and/or other extremities) to become numb and remarkably pallid; this is followed by pain, venostasis, and cyanosis due to ischaemia; finally, the attack ends with the return of bloodflow, marked by a hyperaemic recovery phase during which the fingers appear reddened. In SSc patients with severe RP, chronic ischaemia may result in digital ulcerations and occasionally gangrene. There is evidence that the visceral vasculature of SSc patients is also affected by Raynaud's-like abnormalities and vasospasms, with consequent ischaemia and organ damage. Isolated (primary) RP is very common in the general population (~5-10%): however, its almost universal presence in SSc (~98%), being the first symptom in over 70% of patients, highlights its importance when considering populations at risk of developing the disease: identifying the small proportion of individuals presenting with RP who will go on to develop SSc or some other CTD is a key aim of clinical research and practice. The pathogenesis of RP and SSc may indeed be related, with severe RP being a *formes frustes* of SSc (i.e. an extremely mild variant on the SSc disease spectrum, or an incompletely expressed form of the disease).

Cutaneous involvement

While the pace and extent of skin changes in SSc vary widely, three main phases are recognized: oedema, induration and atrophy. The oedematous phase is characterized by stiff, swollen hands and feet, and the appearance of RP. The phase of induration follows, during which the dermis of fingers and other affected areas becomes thick, shiny, taut and fibrotic; the epidermis thins, the production of sweat and sebum is reduced, loss of hair and skin creases is apparent, and there may be areas of hyperpigmentation. Tethering of skin to underlying tendons and joints often causes contractures and friction rubs, and may severely affect hand function and overall mobility. Skin biopsy reveals an abnormal accumulation of collagen fibres in the dermis. Facial skin is often affected, producing a characteristically expressionless face, with thin lips and small, tightened mouth. After some years the atrophic phase begins, and dermal skin may soften: however, epidermal atrophy, scarring and contractures remain.

Immunological features

In many respects the immune system appears to function normally in SSc, with no increase in the rate of opportunistic infections. However, there are a number of highly unusual features, concerning both cell-mediated and humoral immunity (272).

Activation of certain peripheral blood mononuclear cells (PBMCs) is found, and the balance between their various subpopulations is altered. Skin biopsies indicate a dense inflammatory infiltrate of mononuclear cells (MNCs), which gradually disappears as the disease progresses. The mainly perivascular location of these MNCs suggests that the inflammatory infiltrate and the vascular disorders of SSc may be connected in some way. Both activation of immune cells and vascular degeneration precede cutaneous fibrosis, and thus may be of fundamental importance to disease pathogenesis, as discussed in Section 1.3.

Systemic sclerosis is often described as an autoimmune disease, and autoantibodies recognizing connective-tissue antigens have been reported. In addition, the majority (>90%) of patient sera contain autoantibodies which recognize a limited number of intracellular antigens, and the identity of these appears to reflect disease subtype. Some aspects of autoimmunity may be directly involved in the pathogenesis of SSc, while others may be epiphenomena.

Other immune system alterations found in a significant proportion of patients include the presence of circulating immune complexes, positive tests for rheumatoid factor, polyclonal hyper-γ-globulinaemia, and abnormal levels of certain complement proteins (272).

Involvement of internal organs

Renal involvement

Histopathology reveals luminal occlusion of the smaller renal arteries in the SSc patient with kidney involvement. Fibrosis and necrosis are also seen in the later stages of advanced disease. Scleroderma renal crisis involves an alteration in the renin-angiotensin-aldosterone axis (84), and consists of a sudden onset of malignant arterial hypertension, with the consequence of polyuric renal insufficiency. It typically occurs in those early cases of dc-SSc which display very rapid progression of skin involvement (~20%), and was once the most ominous complication of diffuse disease. However, since the introduction of a class of drugs known as angiotensin converting enzyme (ACE) inhibitors, mortality has been dramatically reduced. Timely administration of such anti-hypertensive drugs is assisted by anticipation of impending renal crisis.

Pulmonary involvement

Lung involvement can be manifested in two different ways in SSc, namely interstitial fibrotic lung disease and pulmonary hypertension.

Interstitial fibrotic lung disease is revealed by X-ray as opaque areas in the lower lung. It occurs in a majority of dc-SSc patients (70%), but is unusual in lc-SSc. Patients exhibit cough and dyspnea on exertion, and crackling rales. There is an inflammatory infiltrate into alveolar interstitia and peribronchiolar tissues early in the disease. This is followed by vascular obliteration, and interstitial fibrosis.

Although pulmonary hypertension is rare in dc-SSc, it may be secondary to interstitial fibrosis. However, in 25% of patients with lc-SSc, pulmonary hypertension occurs in the absence of fibrotic lung disease, usually 10-20 years after disease onset. Again, the most common symptom is dyspnea. The pulmonary arteries may become occluded by subintimal proliferation and hypertrophy. The affected patient typically shows rapid deterioration followed by death.

The two patterns of lung involvement can be difficult to distinguish, and neither is easy to detect. Pulmonary function tests indicate a decrease in diffusing capacity of the lungs in both interstitial fibrosis and hypertension; total lung volume is also lowered, particularly in interstitial fibrosis. There has been only limited success in treating either condition (315). Future treatments will probably be directed at the early asymptomatic stages of pulmonary involvement, before extensive fibrosis and/or vascular remodelling have occurred (325). Identification of patient subgroups at risk of pulmonary defects is therefore desirable.

Cardiac involvement

Only 10% of lc-SSc patients have serious cardiac involvement, while this figure increases to about 15% in patients with diffuse disease. Patchy myocardial fibrosis, mainly affecting the ventricles, is the most common cardiac manifestation of SSc, and is often accompanied by contraction band necrosis. Such patterns of scarring are suggestive of a cardiac RP (84), with episodic ischaemia followed by reperfusion. Cardiac conduction systems are adversely affected by fibrosis, resulting in dangerous arrhythmias.

Gastrointestinal involvement

Involvement of the gastrointestinal tract in SSc occurs due to perivascular fibrosis and smooth muscle atrophy. Oesophageal dysmotility with chronic acid reflux is the most common feature of SSc after skin involvement, and occurs with similar frequency in both limited and diffuse subtypes (~85%). Symptoms may be eased by the use of histamine-2 receptor antagonists. Hypomotility of the small and large bowel may also be apparent as pseudoobstructional disorder, malabsorption and bacterial overgrowth.

Although gastrointestinal disorders are seldom the direct cause of death in SSc, they are a major source of discomfort for many patients, and can significantly affect their quality of life.

Clinical course

Limited cutaneous systemic sclerosis

The lc-SSc patient typically presents with recently acquired oedema of the fingers together with a history of RP, which may have been their only symptom for many years. There then follows an extensive period of very slowly developing skin fibrosis beginning with the hands, feet and face, which does not proceed beyond the elbow, knee or neck. Telangiectases and digital tip ulcers may be seen early, while calcinosis (deposition of calciferous material under the skin) is common later on. Serious internal manifestations can occur, but often take decades to appear, the most important being a sudden onset of isolated pulmonary hypertension, which is usually fatal. Other visceral complications include oesophageal hypomotility, small bowel malabsorption and, in some cases, interstitial lung disease. The 6-year survival rate is ~80%, while 12-year survival is ~50%.

Diffuse cutaneous systemic sclerosis

The most likely symptoms of the dc-SSc patient at presentation are a sudden development of RP, together with swollen, puffy fingers, and in some cases arthritis. Tendon friction rubs and skin thickening of the extremities may already be apparent. Pulmonary, cardiac, gastrointestinal and/or renal abnormalities are often detectable at an early stage. The disease progresses rapidly over the next year or two, with very severe and extensive skin thickening which usually includes the trunk. The pace of these cutaneous changes correlates with the involvement of internal organ systems, and consequently increased mortality. However, about 5 years after disease onset, many survivors experience a gradual softening of affected skin, and, although the state of organ systems already affected continues to decline, new organ involvement is unusual. Six-year survival is ~30%; 12-year survival is ~15%.

Clinical investigation, diagnosis and prognosis

When the presenting symptoms are suggestive of SSc or a related CTD, several modes of investigation are currently used to aid diagnosis. Once a diagnosis of SSc has been made (218), assignation of disease subtype is the key factor in predicting disease severity for the individual patient with early disease.

Some of the investigations described below are also relevant to patients presenting with isolated RP, and can help to detect the minority of such patients at risk of developing a more serious CTD.

Clinical examination

Non-invasive clinical examination of the patient at presentation makes a valuable contribution to both diagnosis and prognosis, particularly in poorer countries. The experience of the examining physician is of the utmost importance in the detection of early disease, as symptoms may be subtly expressed at this stage.

Skin scoring

The presence of characteristic and unambiguous sclerodermatous skin changes at presentation may allow an immediate and quite confident diagnosis of SSc to be made. The assignation of disease subtype may also be possible, based on the extent and pattern of cutaneous fibrosis.

For a reliable prognosis to be made on the basis of skin examination alone, a series of semi-quantitative assessments should be commenced immediately following presentation. Several such skin-scoring systems have been devised, each providing a numerical index of both severity and extent of skin involvement, applicable to both limited and diffuse disease. One widely accepted system with high reproducibility is the Total Skin Score (TSS) (58), based on clinical palpations throughout the body. Ten zones of the body are scored from 0-3 according to the degree of skin thickening: the sum of these scores is the TSS. In a study by Clements *et al.*, an inverse relationship was found between survival time and the TSS measured at study onset, and this proved to be significantly more reliable than classification systems based merely on the distribution of affected skin (58).

Widefield nailfold capillaroscopy

The earliest detectable sign of microangiopathy in SSc is an alteration in nailfold capillary architecture, and abnormalities detected by microscopic examination of nailfold capillary loops can be extremely useful in helping to confirm assessments based on skin findings (215). This cheap and relatively simple technique may be useful in differentiating between limited and diffuse subtypes of SSc. Its most prognostically impressive application, however, is in identifying those few patients with isolated RP who are likely to develop SSc or another CTD (42).

Examination of the nailfold capillaries of SSc patients demonstrates a striking and characteristic enlargement and distortion of capillary loops. The lc-SSc patient shows the "slow pattern" of capillary abnormality, and many giant capillaries are seen. In addition to dilatation, dc-SSc patients also display capillary dropout or destruction, which appears as avascular areas. Serial examination of dc-SSc patients reveals this "active pattern" of destruction. However, the technique has its limitations: these abnormalities can also be seen in other CTDs such as Dm and overlap syndromes, and are occasionally seen in diabetes mellitus, and following trauma (16).

Some patients who appear to have severe, primary RP also exhibit abnormal nailfold capillaroscopy, usually of the slow pattern. Such patients are thought to be at an early stage of evolving SSc: the presence of abnormal capillaries in a patient with primary RP is very suggestive of impending SSc (42).

Bibasilar crackles

Simply by listening to the chest, bibasilar crackles may be detected, pointing to the presence of pulmonary fibrosis.

Patient history

The patient's recollections regarding the timing of onset of RP, oedema and skin thickening are also relevant, as are his or her comments on any other symptoms of a visceral nature, which may point to the involvement of particular organs. For example, if blood is being passed in the urine, renal involvement is indicated.

In those patients presenting with isolated RP, the age at onset of symptoms is particularly important: most cases of primary RP develop before adulthood, while RP secondary to an underlying CTD frequently appears for the first time in the third and fourth decades. Particularly severe cases of isolated RP have also been linked with likely progression to a CTD (for review, (169)).

Physiological tests

There are a number of very useful physiological tests relating to the dysfunction of particular organs. Measurement of oesophageal transit time by quantitative oesophageal scintography helps confirm gastrointestinal involvement. The detection of cardiac conduction abnormalities and arrhythmia's by electrocardiography is indicative of cardiac involvement. An important test relating to pulmonary function is measurement of the carbon monoxide diffusion capacity of the lung (DLCO): an abnormal result indicates pulmonary involvement. Information provided by chest X-ray is also an important determinant of pulmonary fibrosis.

While such techniques are important for the thorough assessment of a particular patient, their prognostic value is limited. Ideally such tests should predict, rather than merely record, end-organ damage – the simple expedient of blood pressure monitoring, for example, is invaluable in the prevention of scleroderma renal crisis by judicious use of ACE inhibitors.

Biochemical markers

Many attempts have been made to correlate the presence of SSc with a variety of biochemical markers, the aim being early diagnosis, determination of disease subtype and avoidance of organ damage.

Renal involvement is frequently detected by changes in several biochemical markers of kidney function, including serum measurements of creatinine, creatinine clearance rates, and the detection of abnormal quantities of protein in the urine.

A significant positive association has been reported between serum levels of the inflammatory cytokine tumour necrosis factor-α (TNFα) and the development of pulmonary fibrosis in SSc patients (130). Further, TNFα values of both lc- and dc-SSc patients were significantly higher than those found in normals (130). Serum levels of a variety of soluble cell adhesion molecules have been found to correlate well with clinical disease activity in SSc patients (122). In the case of intercellular adhesion molecule-1 (ICAM-1), a significant difference between lc- and dc-SSc groups has been demonstrated (175). Meanwhile, significant differences between normals and SSc

patients have been found when plasma levels of the complement protein fragments C3d and C4d were measured (307).

Some markers may have potential for the differential diagnosis of primary versus secondary RP. For example, high plasma levels of β-thromboglobulin, a product of platelet activation, were found only in those RP patients who were in suspected transition to SSc (305), and not in those RP patients with carefully defined primary disease. Von Willebrand factor antigen (an indicator of vascular endothelial damage) has been associated with disease severity in patients with RP, being significantly higher in dc-SSc when compared with normals and patients with primary RP (135).

The main drawback of such markers is that, while average measured values of a particular marker may be significantly different between groups of SSc patients and normals, or between groups of patients with diffuse versus limited SSc, there is often a rather wide distribution of values within each group, and/or considerable overlap between groups. For this reason measurement of such markers in a single individual may have little meaning in terms of prognosis or diagnosis for that particular patient, and few of these markers have been appropriate or reliable enough for routine clinical use. Nonetheless, such work has contributed greatly to an understanding of disease pathogenesis.

Autoantibody status

Anti-nuclear antibodies

The detection and identification of particular SSc-specific anti-nuclear antibodies (ANAs) in the serum of an individual patient is one of the most useful diagnostic and prospective guides available: ANAs appear early in the course of SSc, and several are highly disease specific. Furthermore, the presence of a particular class is not only indicative of disease subtype, but also predictive of future patterns of visceral involvement, even in the absence of definitive skin findings, since the particular ANAs found in SSc tend to segregate with clinically homogeneous disease subsets.

The presence of ANAs in the sera of patients with isolated RP is unusual and, when present, they are usually of low titre. Thus, the detection of high-titre ANAs in these patients is highly suggestive of a developing CTD, and the specificity of such autoantibodies can have prognostic value (168).

The substantial contribution of autoantibody identification to diagnosis and prognosis in SSc is reviewed in detail in Section 1.8.

Other autoantibodies

Besides autoantibodies which recognize intracellular antigens, a number of other autoantibodies have been associated with aspects of disease expression in SSc. For example, a positive correlation has been demonstrated between the presence of RP and the presence of specific antimyenteric neuronal antibodies in SSc patients (147). Antibodies recognizing interleukin-6 (IL-6) have been detected in SSc patients at a frequency significantly higher than in normals, being particularly common in those

patients with limited disease (337). The occurrence of anti-endothelial cell (EC) antibodies has also been found to be significantly more common in SSc patients (particularly those with dc-SSc) than in normals, being found to coincide significantly with severe digital ischaemia and pulmonary arterial hypertension (242).

Whatever the target of the autoimmune response in SSc, inappropriate activation of the normal adaptive immune response is implied. Adaptive immunity, tolerance to self, and the subversion of these processes in SSc are discussed below. Possible relationships between the aetiopathogenic changes of SSc, and the accompanying immunological findings are also considered.

1.2 ADAPTIVE IMMUNITY

An ideal immune system would have evolved to cause destruction of non-self material whilst avoiding damage to the self. Thus, stimulation of the adaptive immune system is normally caused only by foreign, non-self antigens. This is not a straightforward feat, however, since the immune system receptors which are required to discriminate between self and non-self are inherited quite independently from the self antigens they are required to protect.

Maintenance of a healthy condition of self-tolerance is partly a dynamic, ongoing process, and many regulatory mechanisms are thought to be involved. Failure of any one mechanism of self-tolerance can lead to the body's adaptive immune system becoming capable of recognizing its own cells and molecules, and this is the process of autoimmunity.

The adaptive immune system

Cells of the adaptive immune system

The adaptive immune system consists of two main types of lymphocyte: thymus-derived lymphocytes (T cells) and bone marrow-derived lymphocytes (B cells). The T-cell group is further split into two distinct subsets, T helper cells and T cytotoxic cells (Th and Tc respectively).

The B-cell lineage is responsible for producing and secreting antibodies (immunoglobulins (Igs)), a process which occurs only after a B cell divides and differentiates into a plasma cell. Millions of different B-cell clones are each capable of producing a particular antibody specificity which can recognize a particular set of foreign antigens. The recognition and binding of these foreign antigens by the antibody's specific binding site facilitates their clearance by phagocytic cells, which bear Ig-binding surface receptors. Phagocytic cells, such as macrophages, are

responsible for taking in foreign particles or microbes and breaking them down enzymatically.

A key function of Th cells is to help B cells divide, differentiate and produce antibodies. Thus, both B cells and Th cells are important in promoting the removal of extracellular foreign material. Meanwhile, Tc cells are responsible for the elimination of virally infected host cells, a process which also requires help from Th cells. To enable specific recognition of individual antigens, both types of T cell express T-cell receptors (TCRs). Millions of different T-cell clones are present in the body, each expressing a particular TCR which recognizes a particular peptide antigen. However, for effective T-cell recognition, the peptide must be presented on the surface of a host cell, in association with a major histocompatibility complex (MHC) molecule. In the case of Th cells, this relies on the actions of specialized antigen presenting cells (APCs), of which there are several types, including dendritic cells (DCs), some types of macrophage, and, under certain circumstances, B cells.

Activation of helper T lymphocytes

All APCs express the MHC human lymphocyte antigen (HLA) Class II molecule, and ingest material from their extracellular environment. This material is broken down enzymatically in the acidic lysosomes, where the resulting peptides are thought to associate with the MHC Class II molecules (this is known as the exogenous pathway of antigen processing) (239). Each peptide-MHC Class II assembly is then presented on the surface of the APC. Meanwhile, Th cells express the CD4 co-receptor, which is capable of specifically recognizing and binding to MHC Class II. For an effective APC-Th cell encounter, a membrane-bound MHC Class II molecule bearing a peptide antigen must be recognized by an appropriate antigen-specific Th cell bearing the CD4 co-receptor. To facilitate this interaction, each TCR is physically associated with a CD4 molecule on the Th cell surface. The TCR complex also includes a CD3 accessory molecule, which is vital for signal transduction. The sum total of these interactions is known as Signal 1. However, a further requirement for successful Th cell stimulation is interaction of co-stimulatory molecules i.e. Signal 2. The CD28 molecule on the Th cell surface must bind with either a B7.1 (CD80) or a B7.2 (CD86)molecule on the APC surface. Only when both Signal 1 and Signal 2 are fulfilled will Th cell activation occur. During activation of a Th cell, a cytokine signal (IL-1; lymphocyte activating factor) is passed from the APC to the Th cell. In turn, the IL-1-activated Th cell releases IL-2 (T-cell growth factor) and also assembles IL-2 receptors (IL-2Rs) on its surface. The consequence is IL-2 autocrine stimulation, and clonal proliferation into many activated Th cells, each having the same TCR specificity.

Activation of cytotoxic T lymphocytes

The MHC Class I molecule is expressed on the surface of all nucleated human cells, and presents peptides resulting from proteolysis of internally derived proteins (the

endogenous pathway). All Tc cells express the CD8 co-receptor which is capable of specifically recognizing and binding to MHC Class I. The Tc-cell TCR complex consists of the TCR, the CD8 co-receptor and the CD3 accessory molecule. For effective Tc cell activation, a membrane-bound MHC Class I molecule bearing a peptide antigen must be recognized by an appropriate antigen-specific TCR complex. Other cell adhesion molecules present on the surfaces of the Tc cell and on the target cell function to increase the binding affinity of this interaction, and these may include the costimulatory molecules mentioned above.

For full activation, however, the Tc cell also needs help from Th cells which recognize the particular viral peptide concerned (181). Due to the inevitable presence of viral particles in the extracellular environment, specific Th cells will previously have been activated following recognition of virus-derived peptide antigens presented by APCs. The activated Th cell stimulates the Tc cell via the production of IL-2. Following Tc-cell stimulation, clonal proliferation of Tc cells takes place. The resulting activated Tc cells are then capable of binding to similar virally infected cells, which is followed by the release of cytotoxic substances aimed at enzymatic destruction of the infected cells.

Activation of B lymphocytes

A further function of Th cells is in the activation of B cells. All B cells express their own particular type of Ig molecule on the cell surface. The Igs frequently recognize and bind to intact antigens i.e. they are capable of recognizing tertiary protein structure. The first step in B-cell activation involves interaction of the surface Ig with specific antigen. Such interactions are promoted by a particular type of APC. Follicular DCs are found in spleen and lymph node follicles, and express receptors for immune complexes containing complement and antigen. They are thought to accumulate and hold unprocessed antigens on their extracellular surfaces, which are then available for specific stimulation of appropriate B cells via their membrane-bound Ig receptors (179). Due to the constant recirculation of lymph fluid through the spleen and the lymph nodes, an appropriate B cell is likely to come into contact with an APC which is binding an immune complex which includes a particular antigenic particle. Following specific B-cell recognition, the APC releases IL-1 which causes activation of the B cell (clonal selection).

Following activation, B cells are ready for Th-cell help. Depending on the specificity of the Ig on the surface of the B cell, specific antigenic particles are ingested, processed and presented as peptide antigens in association with MHC Class II molecules. Activated Th cells recognize the recently stimulated B cell via an appropriate specific TCR complex interaction with the MHC Class II-bound peptide on the B cell surface. Again, co-stimulatory molecules are also vital: the CD40 receptor on the B cell surface specifically binds with the CD40 ligand. This ligand is upregulated on activated Th cells. Once an appropriate antigen-specific Th-cell encounter takes place, the Th cell

will produce cytokines that further stimulate the B cell (IL-2, IL-4 (B cell growth factor), IL-5, IL-6 (B cell differentiation factor), and interferon-γ (IFNγ)). These cause the B cell to proliferate and differentiate into a clone of plasma cells capable of producing vast quantities of soluble Ig.

It is thought that B cells may also function as APCs, and present peptide antigens to naive Th cells. To aid this process, it is believed that follicular DCs can induce upregulation of MHC Class II and co-stimulatory molecules on resting B cells, thus promoting their effectiveness as APCs (179). However, B cells only ingest the antigenic particles which are specifically recognized by their surface Igs. These particles will have other antigenic regions which are not recognized by the particular B cell concerned. The peptides making up these other antigenic regions will, however, be processed and presented by the B cell. Consequently, the presentation of antigens to Th cells by B cells is believed to be an important aspect of the spreading and maturation of the immune response to different epitopes of a single protein antigen, and is known as intermolecular help (see Section 1.7).

1.3 PATHOGENESIS

A satisfactory model of SSc pathogenesis would include vascular, fibrotic and immune aspects of the disease. Understanding in each of these areas has increased greatly in recent years, but interrelating them into a coherent whole remains difficult, particularly while knowledge of the initiating step is lacking. Although the various hypotheses vary in detail, a basic model of current thinking is described below (for reviews see (105,196,199,265,318,320)).

The culminating feature is excessive deposition of connective-tissue matrix proteins by scleroderma fibroblasts (FBs). However, this appears to be preceded by vascular endothelial injury/activation and a perivascular infiltration of activated MNCs. Autoimmunity may be a cause and/or an additional consequence. A number of different cytokines, growth factors and cell types are doubtless involved in the pathogenesis of SSc, and their myriad interactions are the subject of intensive current research.

Vascular endothelial injury and activation

The vascular EC is believed to play a central role in the pathogenesis of SSc (40,165). Indeed swelling, dysfunction and degeneration of the microvascular endothelium may be the earliest manifestations of the disease. Together with platelet products and neuropeptides, EC products control vascular tone (164). Disruption to this delicate three-way balance is thought to underlie the pathogenesis of RP in SSc, and there is certainly evidence to support this. Plasma levels of endothelin-1, a potent local vasoconstrictor and a product of ECs, are raised in both primary RP and SSc (166,370), while release of the vasodilator endothelial-dependent relaxation factor is deficient.

A further role of the endothelium is in the control of vascular permeability (for review see (263)), and the presence of oedema in early SSc reflects its aberrant function. There are signs of mast cell activation in scleroderma (155), and high concentrations of mast cell-derived histamine are found in SSc sera. Histamine is a vasoactive amine which acts on ECs to cause increased capillary permeability.

Increased expression of cell adhesion molecules by ECs is indicative of a state of endothelial activation. Both ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) have been shown to be upregulated in the vascular lesions of SSc (321). The cause of this EC activation is unknown, but may be related to endothelial damage.

There is evidence that the vascular endothelium becomes physically injured in SSc, with a certain amount of cell death. Increased serum concentrations of β-thromboglobulin and circulating platelet aggregates imply platelet activation, and this is thought to be a direct consequence of endothelial damage. The raised levels of Factor VIII (von Willebrand factor antigen) found in SSc plasma are also consistent with endothelial destruction, since Factor VIII is known to be sequestered in EC granules.

The exact mode of assault on the vasculature is unknown. Several different factors may act singly or in concert. In-vitro studies have shown that a majority of SSc sera are directly cytotoxic to ECs by a non-immune mechanism, and the soluble protein responsible may contribute to vascular damage *in vivo*. In severe cases, RP may itself lead to endothelial injury, and free-radical species resulting from repeated ischaemic episodes have been implicated. Involvement of the complement system has also been suggested. Alternatively, environmental agents may be responsible. In recent years, immune theories of endothelial activation and injury have become increasingly attractive.

Whatever the initial causes of dysregulation and damage, the result is a more permeable, activated endothelium with physically disrupted components. Activated ECs are metabolically profligate, producing increased quantities of IL-1, IL-6, basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF). Meanwhile, activated platelets release PDGF and transforming growth factor β (TGFβ). The upregulated EC adhesion molecules ICAM-1 and VCAM-1 function to attach activated leucocytes to the vascular endothelium in preparation for extravasation, and any increase in microvascular permeability improves access to the interstitium for both PBMCs and cytokines (268). A likely consequence of a damaged, more permeable endothelium is to make components of the basement membrane more exposed on the endothelial surface: indeed, fragments of EC-derived connective tissue may be adrift in this inflammatory milieu.

Taken together, these factors create an environment which may provoke an immune attack on the vascular endothelium itself. Several interrelated mechanisms of immune-mediated vascular damage have been suggested, and will be discussed presently.

Immune system activation

While there is plenty of evidence supporting activation of the immune system in SSc, the initiating cause is unknown, and it remains uncertain which aspects occur before, after, or simultaneously with the vascular lesions. The complex relationship between vascular and immune events in SSc is a particularly unclear and controversial subject. As LeRoy has said of SSc pathogenesis, '...cause and effect have not been sorted out...', continuing, '...a cycle of interactions seems plausible, inviting further study of what comes first.' (197). On the basis of present knowledge, it would appear that a destructive cycle of vascular injury is set up in which immune cells probably have a key role.

Distribution of activated immune cells

In SSc, while the total population of peripheral T cells appears to be normal, analysis of subpopulations reveals an increase in the ratio of Th cells ($CD4^+$) to $CD8^+$ (suppressor/cytotoxic) T cells. Meanwhile, the B cell:T cell ratio is normal. Activation of these Th cells is implied by a higher proportion of T cells expressing the IL-2R, and by elevated serum levels of soluble IL-2 and IL-2R, which appear to correlate with disease activity, severity and mortality. Other products of T cell activation such as IL-4, IL-6, TNFα and soluble CD4 antigen are also raised in SSc sera, and the expression of protooncogenes such as *c-myc* by peripheral T cells is upregulated. Further, the increased concentration of serum IL-1 found in SSc is consistent with Th cell activation by APCs. As well as α/β T-cell activation, recent results point to an important role for T cells carrying the less common γ/δ TCR: the prevalence of $V_{\delta 1+}$ T cells is significantly increased in patient sera, and they too appear to be in an activated state (113).

A perivascular inflammatory infiltrate of MNCs around microvessels of the reticular dermis and between the dermis and the subcutaneous tissue is seen early in SSc, and consists mainly of activated Th cells and activated monocytes (macrophages) (93,178), including a significant accumulation of $V_{\delta 1+}$ T cells (113). Co-localization of cytokines and Th cells has been demonstrated in the perivascular lesions of SSc, and implies involvement of activated Th cells in endothelial events.

Adhesion molecules and homing of PBMCs to the vascular endothelium

Lymphocyte homing is mediated by cell adhesion molecules. Circulating lymphocytes expressing appropriate and sufficient cell adhesion molecules (homing receptors) are known to home to high endothelial venules where they bind to complementary addressins. These addressins are the EC adhesion molecules ICAM-1 and VCAM-1, upregulated by EC activation in order to provide enhanced adhesion to passing activated lymphocytes (321). The natural ligands of ICAM-1 and VCAM-1 are lymphocyte function-associated antigen-1 (LFA-1) and very late antigen (VLA), respectively. The LFA-1 ligand is found on lymphocytes, monocytes and natural killer (NK) cells, while VLA is widely expressed on leucocytes except neutrophils (for reviews see (191,221,323)).

In-vitro studies have shown that a subfraction of activated PBMCs from SSc sera display abnormally enhanced binding to ECs. Furthermore, the perivascular infiltrate of MNCs in SSc-affected skin display increased LFA-1 and abundant VLA expression. In the context of increased vascular permeability, this is sufficient explanation for the perivascular infiltrate of activated PBMCs seen in SSc. Recent evidence specifically suggests homing of $V_{\delta 1+}$ T cells to perivascular sites of inflammation in the early oedematous phase of SSc (113).

Types of immune response in SSc

Thus, several aspects of the immune system and various types of immune cell may be involved in the pathogenesis of SSc: the infiltration of activated Th cells and macrophages to perivascular sites of inflammation has been noted; the presence of autoantibodies in SSc sera implies a role for B cells; in addition, there is evidence of a MHC-independent cytotoxic immune response, and this has been associated with γ/δ T cells and NK cells. These responses are discussed in turn below.

Delayed type hypersensitivity

Thus far, the scenario described above resembles a delayed-type hypersensitivity response. As discussed earlier, this involves TCR recognition of processed antigenic peptides bound to MHC Class-II molecules on the surface of APCs. Consequent activation of the Th cells causes release of IL-2, IFNγ and TNFα, followed by Th cell proliferation, one consequence of which is macrophage activation. A hypothesis has been developed for SSc which proposes an autoimmune response to components of the vascular endothelium, possibly made available as a direct or indirect result of vascular injury. A basic requirement for such a hypothesis would be a breakdown of self-tolerance mechanisms: this aspect will be discussed separately in Sections 1.4 and 1.7.

The target of endothelial immune injury may be the EC itself or its basal lamina, which comprises several connective tissue components. There is evidence that connective tissue components can cause T cell activation in SSc – recirculating Th cells recognizing laminin and type I collagen have been demonstrated in patient sera. Peripheral blood MNCs from patients with SSc have been shown to undergo blast transformation when cultured in the presence of type IV collagen and laminin (150), both components of the endothelial basement membrane, and it was suggested that lymphocytes may become activated at sites of endothelial injury (150). When PBMCs were taken from patients with early SSc, increased production of IL-2 by $CD4^+$ cells was similarly demonstrated following incubation with type I collagen (132).

It was mentioned previously that a soluble cytotoxic factor was present in many SSc sera, and this has been identified as the protease granzyme-1. This enzyme is a product of the granules of activated Tc cells and is capable of disrupting basal lamina due to its type IV collagenase activity, leading to production of type IV collagen and laminin fragments. Granzyme-1 activity may thus be an important factor leading to the

production of an MHC-dependent autoimmune response to components of basal lamina in SSc (197). Evidence for the MHC-independent activation of Tc cells in SSc will be described later.

The identity of the particular population of APCs involved in the purported presentation of EC antigens *in vivo* is unknown. Possible mechanisms involved in Th-cell activation are described presently: each may be important at a different stage of the immune response.

Consequences of Th cell activation

The activation of Th cells by APCs would lead to release of IL-1 by the APC and consequent proliferation of specific T-cell subpopulations with further cytokine production, and inflammation. The initial product of Th cell activation in delayed-type hypersensitivity is IL-2, which has paracrine and autocrine effects on antigen-activated T cells causing clonal proliferation. Other cytokines released by Th cells following IL-2 stimulation include IFNγ and TNFα.

Endothelial cells become activated in the presence of TNFα, causing an increase in the endothelial expression of ICAM-1 and VCAM-1. In addition, TNFα causes the release of vasodilatory substances by ECs. Together, TNFα and IFNγ increase microvascular permeability. This leads to EC recruitment of monocytes and lymphocytes from the blood.

Class-II MHC expression on ECs and monocytes is upregulated by IFNγ, causing more efficient antigen presentation at the local site. In addition, macrophages in the immediate vicinity of IFNγ become activated. Activated macrophages in turn produce a variety of cytokines and growth factors, including IFNγ, TNFα, TGFβ, IL-1β and PDGF. If endothelium-derived antigens were being presented in SSc, a self-perpetuating cycle of endothelial destruction could be set up. Again, it should be pointed out that the inciting event has not yet been identified.

Antigen presentation by dermal macrophages

Connective-tissue fragments from EC damage could be processed and presented by dermal macrophages and presented to T cells which coincidentally entered the tissues from the bloodstream. An appropriate antigen-specific encounter, with consequent T cell activation and TNFα release, would result in, or contribute to, EC activation. As a consequence of local changes to the vascular endothelium, other antigen-specific T cells would then be more likely to reach the site and join the reaction. This amplification mechanism via EC activation may be an important component of the normal delayed type hypersensitivity response (1).

Antigen presentation by endothelial cells

Infiltrating MNCs' production of IFNγ causes enhanced Class II MHC expression on ECs, and renders them capable of antigen presentation. Connective tissue fragments

produced by EC damage could thus be presented to T cells by ECs. In this way ECs could modulate T cell responses, giving help for IL-2 generation by T cells with consequent T cell proliferation.

Antigen presentation in lymph nodes

Transport of fragmented connective tissue molecules to lymph nodes is also plausible. Here, antigens are processed and presented by the resident APCs and there is an increased likelihood of a chance encounter with an appropriate T cell. Following antigen-specific Th-cell activation, clonal proliferation with increased expression of Th-cell adhesion molecules would follow. In turn, these factors lead to an increased chance of an appropriate encounter at the peripheral site.

Co-stimulation by connective tissue fragments

The VLA receptor is known to be capable of direct binding to a variety of connective tissue components (191). Exposure of basement membrane antigens such as laminin, fibronectin and type IV collagen may result in direct binding of these extracellular connective tissue matrix proteins to T cells via T-cell VLA. Together with a different MHC Class II-bound antigenic stimulus engaging via the T-cell antigen receptor, connective tissue proteins could perform a costimulatory role in T cell activation (for review (196,318)).

Humoral immune system

Pathogenic anti-endothelial cell antibodies

The presence of autoantibodies recognizing connective tissue components laminin, and collagen types I, III, IV and VI has been demonstrated in some SSc sera (285), and anti-EC antibodies are also found in ~30% of SSc patients (141). This suggests that activated autoreactive Th cells which recognize connective tissue fragments may give help to corresponding autoreactive B cells, promoting differentiation into plasma cells and subsequent autoantibody production.

Anti-EC autoantibodies, as already mentioned, occur in ~30% SSc sera. Recent evidence suggests that these particular antibodies could actually be involved in SSc pathogenesis (29,266). Anti-EC antibodies were shown to induce monocyte adhesion to vascular ECs *in vitro* coincident with increased expression of ICAM-1, VCAM-1 and E-selectin on ECs (45). The antibodies are of the IgG family, and bind to an unknown site on the EC via their antibody-binding domain (F_{ab}) fragment. This induces activation of the EC, and upregulation of cell adhesion molecules, possibly by an autocrine IL-1 effect (45). Some believe that anti-EC autoantibodies recognize a particular EC surface antigen (45). Others consider that the anti-EC autoantibodies of SSc sera recognize a particularly heterogeneous range of EC surface antigens, and it has been speculated that these may include intracellular antigens abnormally expressed at the cell surface (141).

Autoantibodies recognizing intracellular antigens

Autoantibodies which recognize intracellular antigens have been found in up to 97% of the scleroderma population. This is a rather more puzzling aspect of the SSc disease process, and some have dismissed it as a mere epiphenomenon, with no significance to disease pathogenesis (333). There is evidence that the production of these autoantibodies is also mediated by autoreactive T-cell help, implying that a breakdown in both T- and B-cell self-tolerance to certain intracellular antigens is involved in SSc. The expression of intracellular antigens on the surface of ECs mentioned above is one possible explanation of how normally sequestered self-antigens are made available to the immune system in SSc: others are discussed in Section 1.7.

Cell-mediated cytotoxicity

What is described as cell-mediated cytotoxicity encompasses the functions of at least three different cell types. Each type of cell-mediated cytotoxic response has its own system of receptor/ligand interactions for binding the cytotoxic cell to its target. First there is T_C cell killing, which is important in the removal of virally infected cells, and, as discussed earlier, this involves TCR recognition of processed antigens bound to the MHC Class I molecules of the target cell. There is no evidence of this kind of activity in SSc. Secondly, there is killing by activated NK cells. A third type of cell-mediated cytotoxicity is carried out by killer (K) cells.

It has been mentioned that the $V_{\delta 1+}$ T cell population is increased in SSc. The γ/δ T-cell is not fully understood: while it is thought that both cytotoxic and helper subpopulations exist, the cytotoxic subset may predominate. This would seem to imply a role for typical Tc cell functions in SSc: however, only NK and K cell functions are thought to be important in SSc pathogenesis.

Natural killer cells

Natural killer cells are lymphocytes which express neither T cell or B cell-specific receptors. They bind to the target cells in inflammatory sites via the cell adhesion molecules discussed earlier and kill by direct cell-mediated injury. The NK cell is thought to be activated by Th cell-derived IL-2 in the inflammatory site, after which it becomes the functional lymphokine-activated killer cell (LAK), poised for non-specific local attack. It is believed that some Tc cells are capable of LAK-type killing – in other words a cytotoxic response not involving specific antigen recognition by their TCR.

In SSc there is a low level of NK cells in the circulation while there is an accumulation of these cells in the perivascular inflammatory lesions. Recently a much higher than normal proportion (50%) of the $V_{\delta 1+}$ subset of T cells in SSc patients was shown to co-express CD8, the marker of the cytotoxic T cell. The SSc $V_{\delta 1+}$ T cells were also shown to display enhanced expression of VLA. This and other evidence suggests that $V_{\delta 1+}$ Tc cells may play an important effector role in SSc, involving MHC-

independent, LAK-like cytotoxicity mediated via cell adhesion molecules and possibly directed against ECs (113).

Antibody-dependent cell-mediated cytotoxicity by K cells

Lymphocyte-like K cells have no T-cell antigen receptors. However, they do have receptors responsible for specifically binding the crystalline fragment domain (F_c) of IgG ($F_{c\gamma}$). Normally they are important in the anti-tumour response, killing antibody-coated cells by extracellular mechanisms. The $F_{c\gamma}$ fragment of IgG antibodies binding to their target cell antigen is recognized and bound by K cells. Consequent killing of the target cell by K cells is termed antibody-dependent cell cytotoxicity (ADCC). Rather than being a specific cell type, the K cell may represent a function. Evidence suggests that some Tc and NK cells have Fc receptors, and can kill specifically by the K-type method.

It has been found that normal PBMCs can kill ECs when co-cultivated in the presence of serum from ~20% of SSc patients, and the Ig fraction of SSc sera was shown to be necessary for this effect (146,216). The process is mediated by IgG antibodies recognizing EC surface antigens, apparently by a K-type ADCC response (141,146).

Fibrotic lesions

The fibrotic lesions of scleroderma result from the excess deposition of normal components of connective tissue by a subset of activated FBs, namely collagen types I, III, V, VI and VII, proteoglycans, fibronectin and tenascin (84,162). Concentrations of the corresponding mRNA species are also increased ((176); for review see (70)). A basic FB defect is not responsible since FBs taken from the unaffected skin of SSc patients display normal levels of matrix production (199). Nor is altered catabolism of collagen thought to be a factor: collagenase synthesis of SSc-FBs is normal (24). The SSc-FB goes on overproducing matrix proteins for several passages *in vitro*, and expression of *c-myc* protooncogene is also increased. Cultured SSc-FBs do not respond normally to growth factors (176), and resist the quiescence normally induced by serum deprivation. Thus, there also appears to be a defect of FB growth regulation which is not directly related to matrix secretion (195,196).

Since the activated phenotype of in-vitro SSc-FBs is eventually lost, in-vivo factors are thought to be responsible for their original activation. Histological studies reveal that activated FBs are colocalized with the perivascular MNC infiltrates of SSc. Although the initiating stimuli are not known for certain, soluble mediators from the inflammatory cells present in these lesions do seem to be involved. An alternative source of mediators is the activated EC (75) and, to some extent, the FB itself.

With regard to chemical mediators, TGFβ is a particularly likely candidate: TGFβ is the primary fibrogenic cytokine, activating and stimulating normal FBs to produce connective tissue components. Overproduction of TGFβ by SSc MNCs during the

autologous mixed lymphocyte reaction has been demonstrated *in vitro* (257), while increased quantities of TGFβ mRNA in bronchoalveolar MNCs have been detected in SSc (and SLE) patients with lung involvement (73). A key observation was the in-situ identification of TGFβ and type I collagen mRNA colocalized in the perivascular lesions of SSc skin. Type I and type VII collagen fibrils have also been demonstrated to occur in the same topographical distribution as TGFβ in SSc skin (140,291). A likely initial source of TGFβ in SSc is the infiltrate of activated macrophages, and the platelets, which pass freely through the permeable endothelium to the dermal and subcutaneous fibrotic site. Once stimulated by TGFβ, however, FBs can produce their own TGFβ in an autocrine fashion.

Platelet-derived growth factor may also be important in SSc pathogenesis: it is thought to be a key mediator of TGF-β-directed effects, and has been found colocalized with ECs and macrophages in early lesions. Besides ECs and macrophages, the activated platelets of SSc are a further source of PDGF. Autocrine release of PDGF by normal FBs is also known to be induced by TGFβ. It appears that TGFβ upregulates the PDGF-AA ligand - PDGFαα receptor activity of SSc-FBs, which then respond readily to PDGF by proliferating and upregulating FB matrix production.

Thus, the chronically upregulated autocrine release of TGFβ and PDGF by SSc-FBs explains the altered response to extraneous sources of growth factors displayed by cultured SSc-FBs *in vitro*: the SSc-FB fails to regulate cell growth and matrix production, being directed by unregulated autocrine stimulation (199).

In addition to the damage-related release of endothelin-1 from ECs mentioned earlier, SSc-FBs have been shown to overproduce endothelin-1 *in vitro* (174). This effect may be mediated locally by IL-1β from activated MNCs in perivascular lesions (174). Endothelin-1 appears to be mitogenic to fibroblasts (166), and thus may be a further factor in the fibrosis of SSc. Other postulated promoters of the fibrotic phenotype in SSc include certain products of activated mast cells (39) and the topoisomerase I (topo I) enzyme (77).

Models of systemic sclerosis

The main human model of SSc is chronic graft versus host disease (GVHD), which sometimes follows bone marrow transplantation. This phenomenon can be produced by injection of immunocompetent T cells into an immunocompromized host. A disease syndrome develops which mimics many of the features of idiopathic SSc. The disease results from damage caused to endothelium and epithelium by T cells via cytokine mediators (24), and features include MNC infiltration and tissue fibrosis.

The ideal animal model of SSc would replicate all features of the idiopathic disease i.e. endothelial damage and activation, cutaneous fibrosis, perivascular MNC infiltration of skin, and autoantibodies recognizing the characteristic SSc autoantigens. This would enable in-vivo experimentation to be conducted, possibly leading to greater understanding of disease pathogenesis and an effective treatment for the human disease.

The animal models currently available do not fulfil these exacting criteria, and results obtained from them must be guardedly analyzed. The main animal models are the tight-skin mouse (Tsk) models *Tsk1* (reviewed in (27)) and *Tsk2* (55), and the University of California Line 206 chicken (27).

1.4 TOLERANCE

Mechanisms of self-tolerance and immune regulation

T cell tolerance

Central tolerance

In man, autoimmunity is primarily avoided by clonal deletion (i.e. death) of immature autoreactive T cells in the foetal thymus during the perinatal period (for review (247,352)). This process is known as central tolerance, and depends on self-antigens being present in, or passing through, the thymus. However, some autoantigens do not reach the thymus, and for these alternative methods of tolerance induction are available. Such autoantigens may include certain tissue-specific proteins.

The pre-T cell population is formed in the bone marrow, and, although committed to the T cell lineage, these cells do not yet express TCRs, the co-receptors CD4 and CD8, or the accessory molecule CD3. The pre-T cells migrate to the thymus, which is the main site of maturation for all T cells.

Some time after entering the thymic cortex the immature T cells (thymocytes) begin to express both CD4 and CD8. Processes occurring during the passage of these double positive cells from the thymic cortex to the thymic medulla are largely responsible for the mature T cell repertoire of an individual. The thymocytes divide rapidly, and the initial range of TCR specificities is determined by random TCR-gene segment rearrangements.

Each TCR contains one α and one β chain, and each of these consists of constant (C; i.e. the same on many lymphocytes) and variable (V; i.e. specific to each lymphocyte clone) domains. There are separate α and β gene loci, and these are located on separate chromosomes. Two allelic versions of α and β gene loci are available, each containing a small variety of alternative C-gene coding domains, a wide variety of V-gene coding domains, and several joining (J)-gene coding domains. Ultimately only one α and one β locus are utilized to form the mRNA coding for the TCR of a single lymphocyte. Furthermore, special somatic rearrangement processes take place, involving excision of both DNA and RNA to form α-chain and β-chain mRNAs with random combinations of C, J and V genes. When all possible combinations of all possible rearranged α- and β-chains are considered, an individual could generate up to 10^{10} different TCRs (268). From this extremely large and variable range of TCRs, each thymocyte expresses only one, with individual antigen-binding properties. Division of one such T cell results in

many clones sharing the same TCR. Once the TCRs are expressed on the cell surface, the CD3 molecule may join the TCR complex.

Selection events then take place, which are thought to involve MHC-expressing cortical epithelial cells, and bone marrow-derived non-lymphoid cells such as DCs and macrophages, which may act as APCs. Positive selection ensures that only cells expressing TCRs which can recognize a self MHC molecule (Class I or Class II) are stimulated and allowed to survive, and is known as MHC restriction. Thymocytes with no affinity for self MHC are allowed to die (benign neglect), probably by programmed cell death (apoptosis). This is followed by a process of negative selection, during which self-reactive thymocytes (i.e. thymocytes whose TCRs can bind tightly to presented (self) antigen in association with MHC) are killed by apoptosis, leading to a state of self-tolerance. During these selection processes, the double positive ($CD4^+CD8^+$) cells begin to express only one co-receptor molecule, either CD4 or CD8, forming the helper subset and the suppressor/cytotoxic subset respectively. This is dependent upon which particular MHC molecule is recognized by the thymocyte: thus, $CD4^+$ cells will recognize MHC Class II molecules, while $CD8^+$ cells will recognize MHC Class I. Thus, in theory, only those T cells with TCRs which can recognize a foreign antigen associated with a self MHC molecule (Class I or II) will mature. Following maturation, the cells are released into the bloodstream and peripheral tissues.

Peripheral tolerance

Peripheral tolerance occurs after the mature T cells have migrated from the thymus. The process involves the inactivation (anergy) of autoreactive T cells, rendering them incapable of responding to antigenic stimulation.

As mentioned above, for effective stimulation, the mature Th cell must simultaneously receive two different signals. The first of these depends on specific TCR recognition of a peptide antigen associated with a Class II MHC on an APC. Secondly, the APC must provide co-stimulatory signals in the form of cell surface molecules. When a Class II-associated antigen is presented in the absence of such co-stimulation the T cell becomes unresponsive (anergic). Even when subsequently stimulated by a competent APC expressing the same Class II-associated peptide, the cell will not respond.

Meanwhile, peripheral tolerance of Tc cells could involve a process known as cross-presentation. Cross-presentation involves the induction of $CD8^+$ T-cell responses by bone marrow-derived APCs capable of MHC Class I-restricted presentation. This process requires help from appropriate Th cells. Organ-specific self-antigens may be transported to draining lymph nodes for Class I-restricted presentation (41). In the absence of appropriate Th cell help, this leads to Tc cell deletion (181), a mechanism known as cross-tolerance (134).

Suppressor T cells

Some immunologists believe that a subpopulation of antigen-specific $CD8^+$ T cells is capable of recognizing and inhibiting self-reactive lymphocytes. These suppressor T cells (Ts) may be important in controlling the immune response to antigens which do not reach the thymus. They may function by being capable of activation by antigens in the absence of APCs or MHC molecules.

B cell tolerance

Comparable mechanisms are also thought to affect self-reactive B-cell populations. Deletion of immature self-reactive IgM-expressing B cells may take place in the bone marrow following exposure to self-antigens. However, clonal deletion of B cells appears to be a much less thorough aspect of self-tolerance than the deletion of autoreactive T cells, and evidence suggests a rather large population of self-reactive B cells reaches the circulation in the normal individual. Such cells appear to have been rendered unresponsive to antigenic stimulation by mechanisms of clonal anergy. This form of tolerance is induced by the interaction of self-antigen with the B-cell Ig receptor. If it occurs at an early developmental stage, the Ig receptor expression of the mature B cell may be chronically down-regulated. There is, however, a further source of autoreactive B cells. During the course of an ordinary immune response against foreign antigen, Th cell stimulation of the appropriate B cell results in its migration to a primary follicle to from a germinal centre where proliferation of the particular B cell takes place. One aspect of this Th cell-induced proliferation of B cells is somatic hypermutation of the hypervariable regions of the Ig genes. This results in a population of B cells with slightly different antigen binding regions. In the germinal centre, B cells with high-affinity Ig receptors are selected over low-affinity receptor-bearing B cells by programmed cell death. Nevertheless, some of these selected clones may be autoreactive. However, B cells require multiple signals to enable differentiation into plasma cells. The antigen recognized by the B cell must be bound to the B cell's surface Ig, and T-cell help must be available via binding of the TCR to MHC Class II-bound antigenic peptide on the B cell surface. Interactions of cell-surface adhesion molecules on the T and B cells are also important. The T cell can then release lymphokines to the B cell. In the absence of T-cell help, binding of antigen to surface Ig of the B cell may result in anergy. Thus, the activation of autoreactive B cells is normally inhibited and kept in check by a lack of T-cell help.

Anti-idiotype networks

The Ig or TCR binding site (idiotype) may itself be seen as foreign by the immune system. This may result in the production of an anti-idiotypic immune response, and anti-idiotype autoantibodies (and so on, virtually *ad infinitum*). Anti-idiotype autoantibodies may have a regulatory role in the immune system, perhaps by inhibiting

the activation of the idiotype-expressing lymphocytes, as suggested by Jerne's Network Theory.

1.5 AETIOLOGY: GENETICALLY RELATED FACTORS

Discussed below are a range of factors which have been reported to be responsible for eliciting or promoting the autoimmune features of SSc and/or aspects of the SSc disease process itself. In some cases information relating to SLE has also been included: the two diseases are often found in overlap, and appear to have much in common. Both diseases involve autoreactivity with a restricted group of intracellular autoantigens, and there are further similarities regarding aetiological risk factors and suspected environmental triggers. Furthermore, the literature regarding the aetiology of SLE is considerably larger than that concerning SSc, indeed, it has been described as the model autoimmune disease (63). Nonetheless, the two diseases are usually distinct, and only through studying the nature of SSc itself can a complete aetiopathogenetic model of SSc eventually emerge.

Gender

Many CTDs have a predominantly female bias, including SSc, and a number of theories have been put forward to explain this. These usually offer a hormonally related interpretation, since the discrepancy between females and males (relative incidences of new cases) is greatest prior to the menopause. As reported by Simeon *et al.* (316), the expression of the disease is, nonetheless, similar in both sexes.

Hormonal influences

Possible hormonal contributions to the aetiology of the CTDs have been reviewed by Masi *et al.* (69,217). A variety of types of immune cell have been shown to express oestrogen receptors on their surfaces, and both male and female sex hormones may affect immunoreactivity, including autoreactivity. Recently, for example, it was shown that testosterone suppresses in-vitro anti-DNA antibody production by PBMCs derived from SLE patients (172). High concentrations of oestrogens, as found during pregnancy, may inhibit Ts cells, and increase B cell differentiation (217). Oestrogens are also believed to affect cytokine production levels and vascular cell adhesion molecule expression (69). It has also been suggested that hormonal imbalances during growth and development could predispose to CTDs by causing changes to the immunological and/or vascular systems (217).

Microchimerism

Male foetal progenitor cells have been detected in maternal blood for up to 27 years postpartum, and it was surmized by Lee Nelson *et al.* that pregnancy confers a long-term, low-grade chimeric status (21). Further work by the same group showed that SSc

patients with male offspring had a significantly higher concentration of male (i.e. Y-chromosome-specific) DNA in their blood than did similar women without SSc (244). Furthermore, HLA Class-II compatibility between mother and son was more common in the SSc patients, and this finding was used to support the argument that such microchimerism may be involved in SSc pathogenesis, and that SSc represents a form of chronic GVHD (243,244). The syndrome of GVHD occurs in a minority of bone marrow transplant recipients, and has been likened by some to scleroderma (157). The conclusions of Lee Nelson *et al.* (359) were not supported by others, however, who questioned the methods used in their study, and also pointed out that HLA-compatible organ-transplant and blood-transfusion patients do not actually develop SSc as such. On the other hand, scleroderma-like skin changes are observed in some GVHD patients (18), and at least one study has demonstrated that this particular subset of GVHD patients do sometimes test positive for autoantibodies recognizing topo I or Pm-Scl autoantigens, which are characteristic of idiopathic SSc. Therefore, microchimerism as one possible explanation for at least some cases of SSc in women does warrant further examination.

X-chromosomal inactivation

It has been suggested by Stewart (332) that SLE (and presumably SSc) may actually result from the phenomenon of X-chromosomal inactivation, which occurs in all female mammals. The number of dendritic APCs which a developing thymocyte encounters during its passage through the thymus may be very small. Therefore, in theory, a T cell with TCR-specificity for an antigen encoded by the active paternal X-chromosome (for example) could, by chance, interact only with APCs which had an active maternal X-chromosome (i.e. presented only maternally encoded proteins), thus escaping negative selection. In the periphery, this same T cell could later encounter paternal antigens presented by APCs with an active paternal X-chromosome, which it would recognize as foreign. The T cell could then go on to activate appropriate B cells with the consequence of autoimmunity. In the case of a multisubunit/macromolecular particle, the immune response could spread to other antigens with which the original antigen was physically associated, i.e. intermolecular diversification (88,210) (see Section 1.7); however, only X-chromosome-encoded proteins would be capable of actually inciting such a response. Also, this theory does not explain the occurrence of CTDs in males; nonetheless it is an interesting and novel hypothesis, and may account for some cases of autoimmunity, especially paediatric cases.

Immunological factors

Complement deficiency

Complement deficiency has been postulated as an important predisposing factor to autoimmune syndromes, particularly SLE (10). Complement particles are vitally

important for the efficient clearance of immune complexes (i.e. antigen/antibody aggregates). Following sequential activation and selective fragmentation of the various types of complement particle (C1-C9), the immune complex is solubilized and opsonized with a coating of fragments C3b and C4b. This promotes attachment by phagocytes and subsequent engulfment (10).

There are two similar but distinct C4 loci, known as C4A and C4B, and the C4A and C4B particles have slightly different functions on account of different binding affinities. It has been estimated that 10-15% of SLE patients are C4A null homozygotes, while a further 50-80% have a heterozygous C4A or C4B deficiency (10). Furthermore, it has been reported that 64% of a cohort of 25 SSc patients had one or two C4A null alleles (33).

Interestingly, the C4A and C4B genes are located in-between those coding for the MHC Class I molecules and the MHC Class II molecules. It is thought that some apparent associations between autoimmune diseases and HLA alleles may actually be due to complement deficiency genes which are in linkage disequilibrium with the HLA gene concerned. Specifically, it has been deduced that the association of SSc with the extended phenotype HLA-A1/B8/DR3 is, in fact, due to linkage disequilibrium with the C4A null allele (33).

It has been suggested that a deficiency of C4 particles could result in an inadequate host immune response to particular infectious agents (11,33,154). Chronic infection, and/or excessive inflammation and damage to the infected tissues, could lead to spreading of the immune response to the hosts own antigens i.e. autoimmunity (11).

It has also been established that complement components are also involved with clearing potential self antigens released by dying or apoptotic cells (for review, (43)). Apoptotic cells generate surface blebs which contain a range of antigens normally found in the intracellular environment, and these are known to include the Ro particle, recognized by many SLE sera. Therefore, complement deficiencies may result in an abnormal accumulation of normally intracellular self-antigens, with the result of an autoimmune response (see Section 1.7).

It has further been suggested that complement may be involved in the initial process of B-cell tolerance induction by negative selection in the bone marrow (43). While most of this work has been carried out with regard to the aetiopathogenesis of SLE, the deficiency of complement components in SSc suggests that analogous processes may be found to occur in SSc.

Subset skewing of Th cells

Two different Th cell subtypes have been recognized (Th1 and Th2), and each has different functions and releases different sets of cytokines when activated. Cells having the Th1 phenotype release IL-2, IFNγ and TNFα, and are characteristic of cell-mediated immunity. Meanwhile, Th2 cells characteristically produce IL-4, IL-5, IL-6 and IL-10,

and are involved in B-cell proliferation and differentiation. The two cell types also both have other functions related to Ig isotype switching.

Either the Th1 or the Th2 subset may predominate in different autoimmune diseases, and alterations in the balance of the two populations and their cytokine products may be important in disease generation (54,106) and potential treatment strategies. The mechanism of immune deviation, which causes a deviation away from a Th1- towards a Th2-like response, has been proposed to result in an enhancement of antibody responses, which, in susceptible individuals, may contribute to autoimmune diseases where the Th2 response predominates (317). However, the issue is complicated and unresolved (224). Moreover, both types of response appear to be important in SSc, and cytokines from both types of Th cell are elevated in patient sera, as discussed in Section 1.3.

Restricted T-cell receptor usage

There is patchy evidence, mainly from mouse models, that TCRs with particular V-regions are overexpressed in certain diseases. Some indirect evidence of oligoclonality of TCRs has also been shown for human autoimmune diseases. For example, certain Vα and Vβ alleles have been associated with multiple sclerosis (MS) (for review, (365)).

Race and MHC-related effects

The human MHC locus contains both the HLA Class I genes (A, B and C) and the HLA Class II genes (DP, DQ and DR). Each of the three Class II molecules consists of an α- and a β- chain, while each Class I molecule comprizes only one main chain, also denoted α, which is associated with the smaller β-microglobulin polypeptide. The HLA genes are extremely diverse in the general population, although each individual carries only two allelic versions of each component polypeptide chain. With regard to the HLA-DR molecule, only the β-chain shows significant diversity between individuals, while in the case of HLA-DQ, both the α- and the β-chains are highly polymorphic (190). It has been found that differences between alternative alleles of the same HLA gene often relate to those amino acids which will be situated in the peptide binding groove of the functional HLA molecule (365). The binding grooves of the different allelic forms of a particular HLA molecule vary in their ability to bind a given peptide, depending on its net charge, size and hydrophilicity (330). As such, HLA molecules with different functional properties affect the individual's immune responsiveness to particular antigens, including autoantigens. It therefore follows that certain HLA genes may confer susceptibility to certain kinds of autoimmunity (267,301).

In SSc, a number of HLA alleles have been associated with an increased frequency of the disease (for a review of HLA nomenclature, see (245)). These include the MHC Class I alleles A1, B8 and B35 (25), and the Class II alleles DR1, DR3, DR5, DRw8 and DRw52 (25,111,115,202). Importantly, associations sometimes differ when different populations are examined, particularly when racial origins are noted (188,202).

The HLA associations are especially strong when clinical subsets and/or autoantibody-defined subgroups of SSc are considered. Thus, anti-topo I antibodies have been associated with HLA-DR2, DR5 (DRw11 subgroups DRB1*1101-*1104), DR8 and DRw52 (86,112,202,236,281,327), while the anticentromere antibody (ACA) subset displays a high incidence of DR1, DR4 (Dw13 subtypes, DRB1*0403, *0407), DR5 (DRw11), and DRw8 (25,86,112,282,327). In terms of antinucleolar antibodies (ANoAs), anti-Pm-Scl-positive patients are characterized by the presence of DR3 (DRB1*0301) (112,251) and DQw2 (251), anti-Th RNP have a high prevalence of HLA-DR11 (85), anti-RNA polymerase (anti-RNAP) antibodies have been associated with DQB1*0201 (86), and anti-U3 RNP antibodies have been associated with DRB1*1302, DQA1*0501, and DQB1*0602 or *0604 subtypes (7,279).

Recently, several HLA-DQ alleles have been closely associated with certain SSc autoantibody subsets (86,184,226,281,282,361). This work has prompted some authors to conclude that the previously reported HLA-DR associations of some SSc antibodies are actually due to linkage disequilibrium of certain DR alleles with particular DQ alleles. These HLA-DQ associations have, in some cases, been shown to be so close that they depend on a particular amino acid residue. For example, an uncharged polar amino acid residue (tyrosine) at position 30 of the second hypervariable region of the first domain of the DQB1 molecule has been shown to be present in most anti-topo I-positive SSc patients (281,338,361). An association of ACAs with the presence of a polar amino acid (specifically not hydrophobic leucine) at position 26 of the HLA-DQB1 first domain has also been demonstrated (86,226,282). In the latter study, 100% of ACA-positive Caucasian patients had a polar amino acid at position 26 of DQB1. Therefore, the DR associations of ACAs may be due to linkage disequilibrium of DR5 (DRw11) and some DR4 (Dw13) haplotypes with DQw7, of DR1 with DQw5, and of DRw8 with DQw4. Similarly, the anti-topo I studies were taken to imply that there was linkage disequilibrium of DR2 with DQB1*0602 (DQw6), and of DR5 with DQB1*0301 (DQw7) (281). However, it has recently been suggested that the DR and DQ alleles together control the autoimmune response to topo I in SSc (184).

The different prevalences of HLA genes found in different racial groups are thought to underlie the finding that each racial group contains a different proportion of patients with each particular SSc-specific antibody (189). For example, a much higher incidence (76%) of anti-topo I antibodies has been detected in a Thai population of SSc patients than in an equivalent population of Caucasian Australians (26%) (228). It has also been shown that, in Japanese SSc patients, different DR alleles from those reported in Caucasians confer disease susceptibility i.e. the DR4 and DR8 alleles are very common amongst Japanese SSc patients (188,338). A study by Sato *et al.* (189) demonstrated marked and significant differences in autoantibody responses produced by 275 Japanese, 416 North American Caucasian and 24 North American Black SSc patients, with the predominant autoantibodies detected in each group being anti-U1 RNP, anti-

RNAPs and anti-U3 RNP, respectively. The authors also noted a higher frequency of anti-Ku antibodies in Japanese SSc patients, while anti-Pm-Scl antibodies were absent from this population. To some degree these differences were consistent with the different proportions of dc- and lc-SSc patients in the three racial groups (see Section 1.8). Perhaps the most interesting finding was that, when the patients were subgrouped according to disease subtype, significant differences were still apparent. Thus, when dc-SSc patients were considered, the Caucasian group showed a much higher incidence of anti-RNAP I antibodies (43%) than the other two groups, while for Black dc-SSc patients the main association was with the anti-U3 RNP antibody (50%), and Japanese dc-SSc patients had predominantly anti-topo I antibodies (65%). It was concluded that, to some degree, the expression of particular autoantibody specificities is dependent on race independently of disease expression. Again, the immunogenetic background was convincingly argued as a possible explanation for these findings: e.g. the anti-Pm-Scl antibody response has been associated with the HLA-DR3/DQ2 haplotype, which is common in Caucasians, but rare in the Japanese, and this may explain the lack of any anti-Pm-Scl-positive sera in the Japanese patient group. Meanwhile, in a study by Reveille *et al.* (280), the frequency of anti-topo I antibodies was found to be higher (37%) in a population of 27 North American Black SSc patients than in their 118 North American Caucasian patients (17%).

Such results may have important implications for a proposed aetiology of SSc. However, it is also clear that further genetic or environmental factors are important in SSc aetiology, besides the presence of certain predisposing HLA haplotypes (6). A particular group of Choctaw (Native American) Indians has been reported in whom SSc is especially common (prevalence ~1/213 in full-blooded Choktaws) (6). The patients displayed a remarkable degree of homogeneity in their disease, with mostly dc-SSc and anti-topo I antibodies. While a number of HLA allele associations were found in this study, namely B35, DR2 (DRB1*1602), DQA1*0501, and DQ7 (DQB1*0301), a population of Choktaws in a different location had a negligible incidence of SSc, whilst having a similarly high frequency of these same HLA haplotypes.

The closeness of the HLA associations in SSc have recently been tested according to the following model (86). It was hypothesized that each different case of SSc may emerge as a similar pathological aberration, which is channelled by the individual's HLA molecules into one of several distinct subtypes characterized by certain symptoms and particular autoantibodies, or, alternatively, that SSc may encompass two or three separate diseases, each having distinct pathogenetic origins which determine disease expression (86). The results of the study of Fanning *et al.*, suggested, but did not prove, that the latter hypothesis was the correct one (86). Clearly, however, acceptance of this second hypothesis does not in any way preclude the possibility that these purportedly separate disease groups may further subdivide according to HLA polymorphisms and fine autoantibody specificity.

Defective apoptosis

It has been suggested that patients with SLE have a defect in apoptosis. In one pathway of apoptosis, the Fas ligand (Fas-L) binds to membrane-bound Fas (mFas; CD95), which leads to a sequence of events culminating in apoptosis. (for review, (238)). A number of defects relating to Fas and/or Fas-L may be involved in SLE, specifically concerning the apoptosis of self-reactive T cells during thymic selection, and the removal of activated mature T cells following an effective immune response (163).

For example, it has been found that there is a higher than normal level of soluble Fas (sFas) in SLE patients, and that this may be capable of competitively inhibiting the binding of Fas-L to mFas, with the consequence of inhibiting apoptosis (163). If this resulted in an altered threshold for T cell deletion, autoreactive cells with a whole range of different specificities may be allowed to persist (238). It has also been suggested that apoptotic events occurring in the thymus are an important source of apoptosis-related antigens, and, if insufficient apoptosis occurs in the thymus during early ontogeny, an abnormally large population of T cells which are capable of recognizing these particular antigens will escape deletion (47). Studies using animal models of SLE (e.g. the MRL/Mp-*lpr/lpr* mouse) imply that defects in apoptosis and/or upregulation of factors which promote lymphocyte survival can indeed result in a lupus-like disease, characterized by massive lymphadenopathy due to a reduction in activation-induced T cell apoptosis (83,203,237).

In terms of SSc, the possibility that subpopulations of FBs acquire a reduced propensity for apoptosis has been suggested (238), and is currently being investigated in our laboratory (Mr J.Dixey, pers.comm.). Exciting recent developments include the demonstration that anti-EC antibodies from SSc patients can induce EC apoptosis (29). This finding provides further evidence that anti-EC antibodies may be involved in the pathogenesis of SSc.

Cancer

A significantly higher incidence of malignancies, especially lung cancer, has been reported to occur in SSc patients, and some have suggested a causal link (51,290). An elevated and epitope-rich anti-topo I antibody response has also been found following the development of lung cancer in two anti-topo I-positive SSc patients (182). This may be related to the increased production and activity of topo I in cancer (182). An important finding was that SSc-free lung cancer patients did not have anti-topo I antibodies, and it was surmized that topo I overexpression *per se* is an inadequate stimulus for the breakdown of tolerance to this molecule (182). However, in another study, a high proportion (31%) of patients with hepatocellular carcinoma in the absence of a CTD did have ANAs, including those recognizing NOR-90, RNAP I and U3 RNP i.e. antigens which are specifically recognized by some SSc sera (153).

Other genetic factors

Innumerable genes are involved in the adaptive immune response. Each gene will have a number of polymorphisms in the general population, and some alleles may confer a genetic susceptibility to autoimmune disease. Candidate genes are currently being assessed in a number of different centres using modern microsatellite techniques. Genes of particular interest include those coding for TNFα and PDGF.

1.6 AETIOLOGY: ENVIRONMENTAL RISK FACTORS

Pathogens

Pathogens may be involved in the induction of an autoimmune response and/or in triggering a CTD by at least four different mechanisms (260,284).

Molecular mimicry

Molecular mimicry occurs when there is a structural homology between a self antigen and an antigen present in an infecting pathogen (44,256). This stimulus is thought to induce an immune response that cross-reacts with the self antigen (15). A very short sequence of amino acids may be sufficient to cause such cross-reactivity. A number of viruses have been shown to contain peptides with a high degree of homology with certain autoantigens characteristic of particular rheumatic diseases (374). In terms of SSc, it has been reported that a highly antigenic region of the topo I molecule comprizes six sequential amino acid residues that are identical to a sequence found in $p30^{gag}$ - a group-specific antigen contained in several mammalian retroviruses (222). This report was made all the more interesting by the earlier finding that an adjacent sequence of the retroviral $p30^{gag}$ antigen displayed cross-reactivity with autoantibodies against the 70-kDa polypeptide of U1 RNP, which are found in MCTD and SLE (273).

However, it is important to distinguish between an autoimmune response and a disease syndrome: in some cases, the presence of autoantibodies which recognize a particular self antigen is sufficient to cause symptoms of the disease itself, e.g. antibodies recognizing the nicotinic acetyl choline receptor in myasthenia gravis, while in other diseases, including SSc, the presence of a disease-specific ANA alone is not thought to be directly pathogenic (256). The induction of an autoimmune response by mechanisms of molecular mimicry is discussed further in Section 1.7.

Microbial superantigens

Certain bacteria and mycoplasma have been shown to cause clinical relapses in some autoimmune diseases by inducing polyclonal activation of particular lymphocyte subpopulations via soluble products called superantigens. Superantigens can simultaneously bind to non-polymorphic determinants of Class II MHCs on APCs and to the β chain of certain TCRs without involvement of the antigen-specific binding site.

Each kind of superantigen binds to certain amino-acid sequences expressed by all members of a particular V-gene family. This results in the activation of various T-cell clones having different antigen specificities but sharing a high degree of amino acid homology (67).

Cell lysis and death

It has been suggested that, in some circumstances, autoantibodies are produced as a result of large amounts of cell lysis and death. This could involve destruction of anatomical barriers, an increase in vascular permeability, or the release of normally sequestered, intracellular antigens, with the subsequent engulfment and presentation of autoantigens by macrophages. Such overwhelming quantities of self-antigens could, in turn, make the development of an autoimmune syndrome more likely (15,284).

Subversion of the immune system

It has recently been hypothesized by Pandey & LeRoy (260) that human cytomegalovirus may be involved in the aetiopathogenesis of SSc by mechanisms not directly related to the induction of autoimmunity. The ubiquity of the latent virus amongst the general population was noted, as was its ability to infect ECs and macrophages, to increase the expression of fibrogenic cytokines such as TGFβ, and to induce type IV collagenase expression. It was proposed that, by several different mechanisms, including the down-regulation of MHC Class I molecules, the virus subverts the immune system for its own purposes, establishes a chronic infection of vascular ECs, and induces collagenase and TGFβ activity. The consequence may well be the vascular obliteration and fibrosis characteristic of SSc (260).

Drugs and environment toxins

A very diverse range of chemical substances has been suspected of causing SSc or a CTD resembling it. Occupational exposure to chemicals such as formaldehyde, biogenic amines, epoxy resins, aliphatic and aromatic hydrocarbons has been reported to cause SSc (109), and administration of certain pharmaceutical drugs has also been implicated. A large number of individuals were affected by an SSc-like syndrome termed toxic-oil syndrome following a one-off incident of rapeseed cooking oil contaminated with an aniline derivative. A similar SSc-like syndrome known as eosinophilia myalgia was caused by L-tryptophan supplements which contained the synthetic by-product 3-(phenylamino)alanine (137,173).

Silicosis and silica-induced systemic sclerosis

One fairly well studied example of environmentally induced SSc is the induction of SSc in coal miners and stonemasons, which is thought to be due to silica inhalation. One early report found that Si-SSc could not be distinguished from the idiopathic disease (293). The mine workers studied had all been exposed to silica dust, and 17 had developed Si-SSc. Meanwhile 30 mineworkers who had developed silicosis alone were

also studied, along with a further 22 who had developed neither disease. The miners with Si-SSc had clinical, immunological and serological features which were typical of the idiopathic disease. These features included cutaneous sclerosis in all cases, and a high prevalence of anti-topo I antibodies, oesophageal involvement and bibasilar pulmonary fibrosis. Interestingly, a subset of each of the three groups of miners had evidence of EC damage. Furthermore, ANoAs were surprizingly frequent in the silicosis group (27%), although no SSc-specific antibodies were detected in individuals with silicosis. In a subsequent report by the same group, and involving our own laboratory, anti-Ro/La antibodies were detected in two of the silicosis patients who had no signs of a CTD (227). While the existence of a Si-SSc syndrome has been supported by others (97,295), some studies have found no evidence of SSc amongst persons exposed to silica in the workplace (313).

Silica particles can be ingested by alveolar macrophages, and it is thought that their subsequent IL-1 release causes the FB proliferation and collagen accumulation characteristic of pulmonary involvement (214). Also, in the context of Si-SSc, macrophage-derived TNFα could contribute to lymphocyte proliferation, Ig synthesis and upregulation of MHC and cell adhesion molecules (97). It was also hypothesized that the silica particles may be transported by the lymphatic system, and ultimately may reach the general circulation and be phagocytozed by vascular ECs (293).

It was subsequently shown that the effect of silica on macrophages was specific, since TiO_2 dust did not have a similar effect (98). Clearly, genetic factors are also involved, since only a proportion of silica dust-exposed individuals develop Si-SSc, and this may be on account of particular MHC Class II alleles (227). This effect has recently been demonstrated by Frank *et al.* (97), who showed an association between anti-topo I-positive Si-SSc and the HLA-DR3 allele. This finding was particularly exciting since the anti-topo I-positive idiopathic SSc cases in their study were associated with DR2 and DR5, as has previously been reported (97). The authors concluded that different pathogenic mechanisms lead to the production of anti-topo I antibodies in the two groups, and that a different topo I-derived peptide was presented by MHC Class II molecules in Si-SSc. It was suggested that the presentation of different peptides in the two groups could have been due to (i) the involvement of a different kind of APC (these have been shown to contain different proteases), or (ii) altered antigen processing caused by macrophage-derived cytokines following silica exposure (it is known that TNFα can modulate the pattern of antigen processing by APCs) (97).

An interesting recent finding is that the relative level of sFas:mFas is raised in silicosis patients, but not in SSc patients (345). These findings are reminiscent of those described in SLE earlier. The authors, who had previously found evidence that silica acts as a superantigen, suggested that there may be an accumulation of self-reactive clones in such patients, which, ultimately lead to an autoimmune syndrome (258).

Controversy surrounds purported cases of CTDs (including SSc) resulting from leakage of silicone gel from breast implants. Several studies have demonstrated a high frequency of ANAs in silicone-implanted women, and these included ANoAs, anti-topo I, anti-Pm-Scl, anti-Ro/La and ACAs (68). Others report no increase of SSc in women with silicone implants (37,145,363).

Metal-induced autoimmunity

It has been observed that, in susceptible mouse strains (e.g. the H-2^s strain), chronic exposure to mercuric chloride via injection leads to the development of anti-U3 RNP antibodies by a process requiring Th-cell help (249), and this has become an established experimental model of autoimmunity (120). Possible mechanisms are discussed in Section 1.7. In the context of human autoimmune syndromes, exposure to mercury could occur via dental amalgams or environmental contamination (120).

Physical trauma

Vibration white finger is a condition resembling RP, and appears to arise through damage caused to vascular endothelium by the use of pneumatically powered drills and surgical instruments. An individual with this disorder was reported subsequently to have developed SSc, along with ACAs (60). It is a possibility that such vibrational effects cause tissue destruction and EC disintegration. In susceptible individuals, this could be the trigger for an immune-mediated attack on the vascular endothelium, as was described in Section 1.3.

It is clear that the above-mentioned genetic and environmental risk factors may interact in myriad ways, ultimately leading to the final common pathway of SSc, together with autoantibody production. It seems highly unlikely that all cases of SSc (even all cases of 'idiopathic' SSc) originate from the same combination of propounded genetic and/or environmental risk factors. This is not to say that certain combinations are not more common than others: indeed, the particular manifestations of SSc expressed by an individual patient may reflect the unique aetiopathogenetic mechanisms involved in his or her case (288).

As has been mentioned, in certain organ-specific autoimmune diseases the production of autoantibodies has severe and obvious consequences, which are clearly involved in disease pathogenesis, a good example being the destruction of pancreatic islet cells in insulin-dependent diabetes mellitus. In such cases clinical symptoms can be produced in normal individuals by transfer of the relevant autoantibodies, or by immunization with an appropriate autoantigen. However, the development of autoimmunity is not always so closely associated with clinical disease (54,259). This is particularly true when one considers systemic autoimmune diseases such as SLE and SSc: passive transfer of the anti-DNA autoantibodies of an SLE patient, for instance, does not cause disease symptoms in a healthy person (4). However, there is a

remarkably close association between the presence of particular SSc-specific autoantibodies and disease expression. Research into these connections must surely contribute to a future elucidation of important aetiopathogenic pathways in SSc.

Proposed mechanisms involved in the breakdown of tolerance to autoantigens are discussed below. The nature of autoantigens is then discussed, together with the phenomenon of epitope spreading. The clinical associations of the autoantibodies found in SSc are then reviewed in detail.

1.7 BREAKDOWN OF TOLERANCE AND AUTOIMMUNITY

As Mamula has pointed out, APCs have no inherent prejudice regarding which peptides they will or will not present (210), and both central and peripheral tolerance mechanisms are imperfect. Thus, the normal immune system of a healthy person does contain some autoreactive T and B cells (28), and these remaining autoreactive lymphocytes may escape control mechanisms and become activated. In these circumstances, autoimmunity ensues.

The nature of autoimmunity

Just as in normal immune responses, both T and B cells are required for the production of autoantibodies (186,312). Specifically, it was recently shown that T and B cell collaboration is required for the autoantibody response to topo I in SSc (187). In many CTDs, only a limited number of autoantibodies are produced (125), and these often occur as linked sets, characteristically appearing in the same order in different patients (125,340). Such observations imply that these responses are not polyclonal: autoimmunity in both SLE and SSc is believed to be antigen-driven (87,125). Furthermore, processes which are characteristic of an ongoing immune response, such as Ig isotype switching, somatic mutation, and affinity maturation, are also found to occur as part of an evolving autoimmune response (47).

General features of autoantigens in connective tissues diseases

For unknown reasons it has been observed that many autoantigens share a number of characteristics (for review, (340,341)). First, autoantigens tend to be ubiquitous, and are usually found to occur amongst extremely diverse species, e.g. homologues of the La autoantigen, which is recognized by autoantibodies present in many SLE sera, have been found in amphibians, flies and yeast; the three classes of eukaryotic RNAP (which are precipitated by some SSc sera), have the same basic structure as bacterial RNAP. Secondly, many autoantigens play fundamental roles in cellular biochemistry or architecture, being involved in such functions as pre-mRNA splicing, mitotic spindle formation, DNA binding, and charging of amino-acyl-tRNAs in preparation for translation. Thirdly, there is a very high degree of conservation of the amino acid

sequence of autoantigens. Fourthly, autoantibodies appear to specifically recognize the catalytic centres or functionally important domains of such molecules, which have been highly conserved during evolution.

Consequently, many autoantibodies have been found to be capable of inhibiting biochemical processes and functions. In SLE there is evidence for the direct involvement of some autoantibodies in disease. For example, the deposition of insoluble immune complexes in the kidneys is thought to cause lupus nephritis. However, a directly pathogenic role for SSc-specific antibodies seems unlikely (340): the expression of autoantibodies is not the cause of SSc symptoms, but it is thought to be intimately associated with the disease process, and thus may be an important indicator of aetiology, pathogenesis, and disease expression (340).

The nucleolus as a focus of the immune response in SSc

While autoantigens recognized by SLE sera are mostly located in the nucleus, many SSc autoantigens, including topo I and the centromere proteins (CENPs), have been found to co-localize to the nucleolus during some stage of the cell cycle (94,250). This raises the possibility that the nucleolus is the target of the autoimmune response in SSc (288,340), just as the nucleosome appears to be the immunogen in SLE (125).

Escape from tolerance induction

Several mechanisms have been proposed to explain how an autoreactive T cell could escape tolerance induction.

Failure to present antigen

If a particular self-peptide is not presented by thymic APCs, deletion or anergy of T cells which are capable of recognizing that peptide will not occur. Such a scenario may prevent an autoantigen from being 'seen' by the developing immune system. One example concerns certain organ-specific antigens, which may not be made available to APCs. A further example is the process of X-chromosome inactivation described earlier, which may lead to some antigens not being encountered by APCs in the thymus.

Somatic mutation and forbidden clones

The structure of TCRs was discussed earlier. The rate of random somatic mutations is approximately 10^4 times greater for V-gene base pairs than it is for the base pairs of the remaining spontaneously mutating genes. This amounts to the accumulation of about one base pair mutation per cell division, although some of these will be in flanking sequences and others will not actually alter the amino acid sequence.

These high mutation rates serve to supply additional T and B cell clones to the immune repertoire. For example, the binding of a pathogenic antigen to a TCR complex causes clonal proliferation of that T cell. Some of these multiplying clones may acquire mutations, expressing a TCR differing in a single amino acid. A slightly altered binding site on one such clone may provide even tighter binding for the inciting antigen than the

clone from which the mutation arose (2). At the same time, however, there is the risk of self-reactive clone production, as envisaged by Burnet's forbidden clone theory.

Major and minor determinants

When self-reactive T cells were first shown to be present in healthy animals, it was not understood how they had escaped tolerance, and, further, why such cells were not subsequently activated by self antigens (107). Work by Sercarz and colleagues suggested that not all epitopes of an antigen are equally antigenic. Following immunization with native antigen, some peptides were subsequently found to induce a much stronger T-cell recall response than others. These are known as major and minor determinants, respectively (107). The reason for this discrepancy is that the minor epitopes of a particular antigen are much less likely to be efficiently processed and presented by APCs. Thus, during intrathymic negative selection, the minor determinants are prone to being 'overlooked' by the immune system. Subsequently, the inefficiently presented epitopes of the self-antigen also fail to adequately stimulate the resultant, undeleted, autoreactive T cells. In contrast, major epitopes are characterized by the efficiency of their production by APC processing pathways, and any T cells which recognize them are effectively deleted in the thymus.

Thus, a pool of autoreactive T cells recognizing the minor determinants of self antigens is believed to remain unactivated *in vivo*. They are kept in check by a paucity of the appropriate minor epitopes being presented on APCs. However, stimulation of these cells can occur under particular circumstances: using synthetic peptides, it has been shown that minor determinants which are not efficiently processed and presented by APCs *in vivo*, when introduced from an extraneous source, are capable of stimulating T cells (209).

Activation of T cells recognizing minor determinants by cryptic epitopes

It is believed that the activation of a T cell recognizing a minor determinant is a key moment in the initiation of an autoimmune response, and several scenarios have been proposed which may lead to such an event occurring *in vivo*. The term 'cryptic' epitope is applied to any peptide which is capable of activating autoreactive T cells which recognize minor autoantigenic determinants. Another way of viewing a cryptic epitope is as "an epitope which is not normally generated by APCs at levels sufficient to activate T cells" (15). In its widest sense, cryptic epitopes closely resemble, but need not necessarily be derived from, minor determinants of an endogenous autoantigen. A wide variety of events can lead to the immune system encountering a cryptic epitope, and these are reviewed below.

Modification of self-antigens

Pre-processing of autoantigens

It has been demonstrated that proteolytic events occurring outside the APC, prior to antigen internalization, may affect the peptides produced and presented by the APC (200). Antigens pre-cleaved in an unusual fashion may lead to the production of unique peptide fragments (47), which could act as cryptic epitopes, and such events may therefore be involved in overcoming T cell tolerance.

A subset of autoantigens has been shown to be specifically cleaved during apoptosis (48). These include the 70-kDa subunit of the U1 RNP particle, poly-(ADP-ribose) polymerase (PADPRP) and DNA-dependent protein kinase (DNA-PK) (48-50). Significantly, all these autoantigens are known to be recognized by certain SLE sera. It was proposed by Casciola-Rosen *et al.* that such selective cleavage reactions produce 'immunocryptic' fragments, and, in susceptible individuals, an autoimmune response (48).

More recently, unusual cleavages of certain SSc autoantigens have also been shown to occur, specifically under conditions of oxidative stress (46). The autoantigens involved include topo I and RNAP II. These specific fragmentations, which are only thought to occur in the setting of SSc, require metal ion-catalysis and the generation of reactive oxygen species (ROS). It is believed that such reactions occur during the ischaemic reperfusion events which are a characteristic component of SSc pathology. The specific site is thought to be the nucleolus, since metal ions are known to become localized there. Indeed, an abnormal accumulation of metal ions could be a predisposing factor to SSc in the context of pre-existing RP, with its recurring episodes of ischaemia (288).

It is also possible that high levels of Tc cell activity occurring in SSc lead to a concentrated source of granzyme-1-cleaved autoantigens, which, as mentioned earlier, may involve the generation of connective tissue fragments. There is evidence that granzyme-B causes the production of cryptic epitopes during Tc cell-induced apoptosis (5). Such unusually cleaved protein fragments could be internalized by APCs, resulting in the presentation of novel peptide epitopes. As discussed earlier, in the setting of SSc, there is evidence that, due to EC destruction, fragments of connective tissue-derived proteins are readily available in the inflammatory perivascular micro-environment. Together, these facts could explain the origin of the proposed autoimmune attack on components of the vascular endothelium in SSc.

Oxidative damage to self-antigens

In 1984 LeFeber *et al.* made the intriguing observation that u.v. light-treated cultures of normal human keratinocytes bind autoantibodies derived from SLE sera. The binding of these antibodies was consistent with the externalization of Ro, U1 RNP and Sm antigens onto the cell surface of damaged, but not dead, cells (193). These results were later confirmed by others, who also demonstrated that SLE-derived keratinocytes were

especially prone to u.v. light-induced cell-surface expression of these antigens (116). Subsequent work by Casciola-Rosen *et al.* indicated that sub-lethal doses of u.v. light cause apoptosis of cultured human keratinocytes, and that these apoptotic cells are characterized by surface blebs of ectopically expressed intracellular antigens (47,341). Smaller surface blebs were found to contain endoplasmic reticulum fragments, ribosomes and the Ro antigen, while larger blebs contained Ro, La, nucleosomal DNA and RNPs. It was further suggested that, by reason of their topology, these particular autoantigens were particularly prone to oxidative damage by free radicals during the process of apoptosis (47). Such unusual oxidation reactions may result in structural alterations to these antigens (47).

The exact pathway leading to the development of autoantibodies recognizing Ro, La and U1 RNP antibodies in SLE remains to be clarified. The frequent occurrence of complement deficiency in SLE has been mentioned, as has defective apoptosis, with the likely accumulation of autoreactive T cells, particularly those recognizing apoptotic antigens. Thus, the potential for cryptic determinants of these particular self-antigens to induce an autoimmune response in SLE is clear.

Phosphorylation of self-antigens

The particular range of proteins phosphorylated during stress-induced apoptosis have also been shown to be recognized by a subset of SLE sera (347). This was taken to indicate that phosphorylation occurring during apoptosis lead to the production of neoepitopes (i.e. cryptic epitopes) of self antigens.

Viral proteins in novel packages of self-antigen

The ideas of Rosen *et al.* were subsequently elaborated in a paper describing apoptosis induction by Sindbis virus. It was reported that the viral antigens and the autoantigens co-cluster in the small surface blebs of the apoptotic cells (287). It was suggested that these complexes represent autoantigens occurring in a 'novel immune context' which may lead to the activation of autoreactive T cells in infected individuals (287).

Xenobiotics

The chemical modification of a self antigen by a pharmacological agent or an environmental toxin is thought to be a further possible source of neoepitopes (for review, (121)). Both mercury and gold are known to be capable of forming stable metal-protein complexes by binding to the side groups of certain amino acid residues, thus altering the tertiary structure. Consequently, intralysosomal processing of such proteins by APCs may be altered, leading to the presentation of cryptic epitopes on the APC surface which could circumvent self-tolerance mechanisms. For example, it has been proposed that fibrillarin (the protein component of U3 RNP), is especially prone to mercury-induced modification (270). In the report of Pollard *et al.* (270), mercury was shown to interact with two cyteine residues of fibrillarin, producing a disulphide-bonded form, probably incorporating a mercury molecule as part of the disulphide bridge. Furthermore, evidence was presented which suggested that, while the T-cell

response to fibrillarin was mounted against the mercury-modified form of fibrillarin, the anti-fibrillarin antibodies of these mice recognized the unmodified fibrillarin. This implied that, once T cell tolerance was broken by a cryptic epitope of an autoantigen, an autoreactive B cell response was invoked, which was capable of causing intramolecular epitope spread to other epitopes of the cryptic antigen, and in so doing, propagated a T-cell immune response against the native antigen.

Unusual exposure of normally sequestered self-antigens

Physical damage to the retina of the eye may result in the availability of autoantigens previously not encountered by the immune system. Another such instance may be vibration white finger: it is possible that, in severe cases, vibrational stimuli cause mechanical damage to ECs which results in an accumulation of EC fragments normally only available in small quantities.

Molecular mimics

The role of microbial pathogens in the induction of autoimmune diseases has been reviewed by Behar & Porcelli (15). Microbial antigens which have structural similarities to self-antigens are thought to be capable of inducing immune reactions which cross-react with self-antigens, and this is known as molecular mimicry. The presence of a molecular mimic can lead to the activation of T cells recognizing minor determinants of the analogous self antigen, as described presently.

Altered antigen processing

Exogenous provision of endogenous antigens

One interesting paper has demonstrated that the proteolytic processing of a particular self-antigen differs depending on whether it is endocytosed by, or endogenously synthesized in, the APC. Significantly, it was shown that the process of tolerance induction involves the presentation of endogenously synthesized self-antigens (12).

Heterogeneity in antigen processing by antigen-presenting cell subsets

Several cell types constitutively express Class II MHC, including macrophages and DCs, and are collectively known as professional APCs. As previously mentioned, the B-cell lineage represents a further important APC population.

It has been demonstrated by Vidard *et al.* that not all types of APC contain the same complement of proteolytic enzymes (351). Thus, for a given native antigen internalized by an APC, the array of peptides produced following intralysosomal enzymatic cleavage may differ depending on the lineage of the APC concerned. For example, the processing of ovalbumin appears to differ when carried out by B cells as opposed to splenic APCs, resulting in an alternative profile of presented peptides (351). Furthermore, it was also shown that, under different physiological states, the relative activities of the different enzymes expressed by a particular APC may alter. During tolerance induction in the thymus, a particular population of DCs is thought to be

involved in the process of negative selection of autoreactive T cells (123). Thus, it is possible that antigens processed by non-professional APCs, such as ECs, may lead to the production of cryptic epitopes of a self-antigen. In the context of the wide availability of connective tissue fragments in SSc, together with the apparent upregulation of MHC Class-II molecules on the surface of ECs in the proinflammatory perivascular environment, this provides an additional explanation for the origin of an autoimmune attack on components of the vascular endothelium in SSc.

Other mechanisms leading to autoimmunity

Breakdown of suppressor mechanisms

Any factor affecting the purported populations of Ts cells may result in a rising incidence of autoreactivity.

Superantigens

The potential for polyclonal activation of autoreactive T cells by bacterial superantigens was mentioned earlier (for review, (15)).

Epitope spreading

Thus, autoreactive T cells recognizing cryptic epitopes may be activated by APCs, resulting in their clonal proliferation. Once stimulated, these Th cells will be capable of providing help to appropriate B cells. These will include any B cell which has, via its Ig receptors, recognized, internalized, and processed the whole native antigen, and successfully presented the particular cryptic epitope on one of its MHC Class II molecules. As mentioned earlier, the pool of autoreactive B cells is considerable, but they are normally kept in check by the lack of appropriate Th cell help. Once such help becomes available, a B cell which recognizes an epitope of the native, unmodified autoantigen may be specifically activated. Furthermore, it is believed that the primed Th cells are capable of upregulating co-stimulatory molecules in the B cells – a vital pre-requisite of an effective APC (212). Clearly, a source of native self antigen must also be available. It is significant that B cells only process antigens recognized by their surface Ig, since, when a B cell acts as an APC there is likely to be a rather high concentration of minor epitopes successfully presented on its cell surface. In other words, B cells upregulate the generation of minor self epitopes (15).

Indeed, recent results suggest that, on account of their different antigen processing patterns, B cells may present a much more heterogeneous array of peptides than DCs (108,212). It has been concluded that DCs focus the T-cell response onto certain immunodominant (major) epitopes of an antigen, while B cells subsequently diversify T-cell responses onto subdominant epitopes of the antigen (88,108). It is believed that these B cells can effectively stimulate naive T cells (213), and, under conditions of chronic antigen exposure, effectively spread and diversify the immune response to different epitopes of the same antigen (212).

Furthermore, once initiated, re-stimulation of memory T cells by processing of whole native antigen may occur since less peptide is needed to be presented on the surface of an APC for effective stimulation of a secondary response by memory T cells. Thus, as the immune response develops, minor epitopes of an antigen can become major epitopes.

However it begins, autoimmunity to one epitope of an antigen appears to spread to other epitopes on the same antigen and, ultimately, to other antigens with which the first antigen may physically associate (194,210). This association need not be covalent, and may pass through multisubunit complexes such as the uridene-rich small nuclear RNPs (UsnRNPs). There is evidence that both intra- and intermolecular epitope spreading occur in SLE, and similar mechanisms are believed to operate in SSc. The mechanisms involved are thought to be essentially analogous to epitope spreading involving exogenous pathogens.

Evidence for intramolecular spreading

Experimental results show evidence of epitope spreading in animal models of SLE. Upon immunizing rabbits with an eight-residue peptide from the B, B' antigen (which forms part of UsnRNP complexes), autoantibodies were produced, not only to the immunizing peptide, but to other peptides of the snRNP complex (158). In this study the rabbits even developed a lupus-like illness.

Evidence for intermolecular spreading to non-covalently associated proteins

Other work has shown that immunization of mice with ~100-residue fragments of La autoantigen results in autoantibodies to other areas of the La peptide and, further, to the expression of anti-Ro autoantibodies (283). The Ro-heterogenous nuclear RNP (hnRNP) complexes are known to physically associate with the La protein during certain functional processes.

Conversely, rabbits immunized with short Ro peptides produce autoantibodies to other epitopes of native Ro and, in some animals, an anti-La and anti-DNA response was also found to occur (303). Thus, immunization with short peptides of certain self antigens is capable of breaking down tolerance to entire multiprotein complexes in which that particular amino acid sequence naturally occurs.

Epitope spread in molecular mimicry

Such responses have been studied using foreign versions of self proteins. For example, it has been found that co-immunization of mice with foreign and self cytochrome c results in tolerance to the self molecule being broken (213). Meanwhile, immunization with self cytochrome c alone did not result in autoimmunity. The two forms of the protein have a high degree of homology, with some shared, and some unique, epitopes. It appears that activation of Th cells recognizing an epitope specific to the foreign molecule occurred first, as would be expected. The stimulation of such Th cells would

have made them available to help appropriate B cells. These would have included B cells which had, via their Ig receptors, recognized a cross-reactive epitope of the foreign cytochrome c, and subsequently internalized and presented the various epitopes of the foreign molecule (211). One of these presented epitopes could have been the foreign antigen-specific peptide recognized by the Th cell described above. Thus, this T cell, which recognized a foreign epitope was now able to help a B-cell clone which recognized a cross-reactive epitope. The activated B cell then appeared to have activated naive T cells recognizing common cytochrome c epitopes, which were also present on its surface. This resulted in the breakdown of tolerance to self-cytochrome c (213). This, and other, experiments by the same group, helped establish the currently accepted model of molecular mimicry (211), which may be responsible for the triggering of some autoimmune syndromes.

1.8 AUTOANTIBODIES AND THEIR CLINICAL ASSOCIATIONS

Many CTDs are characterized by the presence in patient sera of particular autoantibodies, some of which are disease-specific (for review, see (205,339,353)). These autoantibodies are sometimes detected before the complete disease syndrome is clinically apparent, for example in primary RP patients destined to develop SSc, or in patients with UCTD who do not yet display sufficient symptoms to identify the particular CTD concerned. Where the physician suspects the presence of a particular disease, this can often be confirmed by routine serological testing in a hospital laboratory. Thus, identification of autoantibody specificities can be important in the diagnosis of individual cases.

A patient's autoantibody profile also aids prognosis in SSc, since some autoantibody specificities are associated with a particular disease subtype, and some are strongly suggestive of certain types of organ involvement. This information allows the clinician to anticipate the most likely complications, and to plan treatments and investigations in a rational manner, with regard to such factors as life expectancy, quality of life, the cost and unpleasantness of certain investigatory procedures, the side-effects of particular drug regimes, and the possible dangers of non-intervention. By advising the patient of the likely progress of their disease, the patient's own views can also be taken into account on these matters.

Autoantibody specificities which are typical of a particular disease but which are not wholly disease-specific do not have the same diagnostic significance, though they may still point to a symptomatic pattern and can alert the physician to possible complications beyond the presenting symptom. In some cases the non-disease-specific autoantibody is in fact characteristic of an overlap syndrome between two types of CTD e.g. anti-U1 RNP antibodies are often found in patients who show some symptoms of SSc, SLE and RA.

It is unusual for a single CTD patient to express more than two or three different autoantibody specificities. Further, where more than one is found, there is a tendency for some autoantibodies to occur together, as discussed below.

Autoantibodies in Sjogren's syndrome and their clinical associations

The Ro and La autoantigens were originally designated SS-A and SS-B, respectively, on account of the very high prevalence of anti-Ro and anti-La antibodies in SS patients (~90% and ~80%, respectively) (3). However, these antibodies are also common in SLE, and are detected in a minority of SSc patients (17,104,143), particularly those who also fulfil a diagnosis of SS (104).

Interestingly, while anti-Ro antibodies sometimes occur in isolation in SS, anti-La antibodies almost always occur in association with anti-Ro antibodies. Despite the fact that anti-Ro antibodies are not disease specific, their presence is closely associated with the same particular symptoms in a variety of CTDs (30) i.e. photosensitive rashes, SS and neonatal lupus.

Autoantibodies in SLE and MCTD and their clinical associations

Four main autoantibodies are characteristic of SLE sera. The anti-Sm specificity is found in ~15% of SLE patients (342), and is virtually disease-specific, while anti-U1 RNP antibodies (~35% of SLE sera) are also closely associated with the so-called MCTD syndrome. The Sm antigens (termed B, B' and D) are core proteins of most U snRNP complexes of which U1 RNP is one example. Anti-U1 RNP antibodies react with U1 RNP-specific proteins of the U1 RNP particle (A, C and the "70-kDa" antigen). In SLE patients, anti-Sm antibodies are rarely seen in the absence of anti-U1 RNP antibodies, though anti-U1 RNP antibodies frequently occur in the absence of anti-Sm antibodies. Anti-Sm antibodies in the context of SLE have been associated with kidney disease and lung fibrosis, while anti-U1 RNP antibodies in the absence of anti-Sm antibodies are indicative of less severe disease. Anti-U1 RNP antibodies have been detected in up to 12% of SSc patients (143). The U2 RNP antibody specificity has also been detected in SLE/SSc/Pm overlap syndromes (66,233), and anti-U4 RNP/U6 RNP antibodies have been reported in a patient with SSc (253).

Anti-Ro antibodies and anti-La antibodies are also very frequently found in SLE sera (~40% and 20% of sera respectively), although it would be considered fairly unusual for a single serum to contain all four antibodies. Just as in SS, anti-La antibodies rarely occur in the absence of anti-Ro antibodies, but anti-Ro antibodies are frequently found alone in SLE sera. The clinical associations of anti-Ro and anti-La antibodies are as described above for SS, although the presence of anti-La antibodies is also negatively associated with lupus nephritis.

Other SLE antibodies include anti-PADPRP antibodies (240,368,369), anti-Ku antibodies, and antibodies recognizing DNA-PK (335). The latter two antibodies have

been found to occur together in some SLE patients. Some SSc and Pm sera also contain anti-Ku (232,234,371), and anti-DNA-PK antibodies (335).

In general, the autoantibody response is less restricted in SLE than in other CTDs, with single sera frequently containing two, three or more autoantibody specificities (339).

Autoantibodies in Pm and Dm and their clinical associations

Many different antibodies have been associated with the occurrence of Pm and Dm (110,275), the most common being anti-Jo 1 antibodies, found in ~20% of myositis sera. Anti-Jo-1 and anti-PL-7 autoantibodies are restricted to those CTD patients with myositis who also fulfil diagnostic criteria for Pm or Dm (231,350) and in whom they are indicative of interstitial lung disease, arthritis, RP, and poor overall prognosis. Meanwhile, the clinical association of antibodies recognizing the Pm-Scl antigen is with the Pm-SSc overlap syndrome. Anti-Ku antibodies have also been associated with Pm-SSc overlap, particularly in Japanese patients.

Autoantibodies in systemic sclerosis

Systemic sclerosis is also characterized by the presence in serum of disease-specific autoantibodies which have been found to be associated with clinical subgroups of disease (for review, see (133,271)). Precipitating antibodies in the sera of SSc patients were first reported in 1963 (14). One of the nuclear antigens uniquely recognized by SSc sera was subsequently identified as a 70-kDa nuclear protein and termed Scl-70 (78), though this was later shown to be a degradation product of a larger antigen found *in vivo* (349). The Scl-70 antigen was eventually identified as being the 100-kDa enzyme topo I (124,311). These early studies established the link between anti-topo I antibodies and the presence of clinical features of SSc as such.

Soon after, the ACA system was detected (20,343), which was also shown to be SSc-specific (52). The component antigens of the centromere which were recognized by ACAs were subsequently identified by Earnshaw *et al.* (82). It was quickly realized that, within the SSc group as a whole, these two SSc-specific antibodies were associated with particular disease subgroups (53,343,349). It is now well established that patients with anti-topo I antibodies tend to have different clinical features from those with ACA specificities. Each of these specificities is found in roughly 25% of SSc sera, and they are generally considered to be disease-specific and mutually exclusive.

The third major class of autoantibodies present in SSc are directed against proteins found predominantly in the nucleolus (177,276,344). The presence of ANoAs in some SSc sera was first reported some time ago using IF methods (14,20,343), and three different nucleolar staining patterns were observed (speckled, homogeneous, clumpy) (20,160). It was appreciated that these probably represented several different nucleolar autoantibody systems (20,343). However, more sensitive techniques were required for the isolation and identification of the autoantigens recognized by these sera (177).

Using IP techniques, antinucleolar antibodies in SSc have been found to precipitate RNAP I (10-15%), fibrillarin (the protein component of U3snRNP) (8%), Pm-Scl (4-17%), ThRNP (4-13%) and, less commonly, nucleolus organizer region protein-90 (NOR-90) (177,251,252,278,286). Rather less data is available concerning their clinical associations, but recent studies are beginning to show that ANoAs are also each associated with particular subsets in the disease spectrum.

While RNAP I is located in the nucleolus, the other two RNAPs (II and III) are found in the nucleoplasm. Although both anti-RNAP I and anti-RNAP III reactivities are SSc-specific, occurring in 10-15% of sera (144,183,277), anti-RNAP II antibodies also occur in SLE and in MCTD/overlap syndromes (297). As mentioned above, a number of other antibodies have been detected in SSc sera, many of which are not disease-specific: these include anti-U1 RNP antibodies (~5%), anti-Ro antibodies (~5%), and anti-Ku antibodies (1-15%).

With the exception of anti-RNAP I and anti-RNAP III antibodies, coexistence of more than one SSc-specific autoantibody in the same serum is generally considered to be very uncommon (327), although isolated cases have occasionally been reported (139,161,185,296).

Clinical associations of autoantibodies in systemic sclerosis

Anti-topoisomerase antibodies

The anti-topo I antibody identifies a subgroup with diffuse disease together with an increased risk of pulmonary interstitial fibrosis (52,102,327,349,357), digital pitting scars (327), malignancies and cardiac abnormalities (357).

Anti-centromere antibodies

Meanwhile, ACAs are associated with a subgroup with limited skin involvement and prominent vascular features (20,53,225,327,343). While ACA-positive patients often have less severe systemic disease (357) and a more protracted disease course (52), a close association between ACAs and severe digital ischaemia has been noted. Furthermore, lc-SSc patients with ACAs are more likely to have telangiectasiae of the digits, and calcinosis than are those lc-SSc patients who are ACA-negative (328).

Anti-RNA polymerase antibodies

Antibodies recognizing RNAPs have been associated with diffuse cutaneous disease, renal involvement, and a very high degree of skin thickening (255,277,278). Other clinical associations reported for anti-RNAP antibodies include decreased survival, joint involvement, and cardiac involvement (185,278). Interestingly, anti-RNAP I-positive patients have been reported to have a low rate of RP early in their disease (278).

Antibodies recognizing the Pm-Scl autoantigen

Anti-Pm-Scl antibodies occur almost exclusively in the Pm/SSc overlap form of disease, which often includes myositis, calcinosis, pulmonary involvement and arthritis (26,208,251,278). This is generally characterized by limited skin involvement and few serious visceral complications, although one report found a high incidence of renal involvement (278).

Antibodies recognizing the Th RNP autoantigen

Anti-Th RNP antibodies (also called anti-To (274)) have been linked with lc-SSc, puffy fingers, poor long-term prognosis (due to pulmonary hypertension), and hypothyroidism (252). The reported association with small bowel involvement (252) has not been confirmed (85). In other respects, the clinical features of anti-Th RNP-positive patients resemble those found in the ACA group, with a low incidence of renal and cardiac involvements (85). Anti-Th RNP antibodies are SSc-specific.

Anti-fibrillarin antibodies

The anti-U3 RNP antibody specificity has been found to recognize the protein component of U3 RNP (201). Anti-U3 RNP antibodies are also SSc-specific, and have been associated with skeletal muscle involvement, primary pulmonary arterial hypertension, and small bowel disease (7,35,254,294). One group has found an association with renal disease and cardiac involvement (7). A particularly high proportion of Black patients have been found to express this autoantibody.

Antibodies recognizing the nucleolus organizer region

The anti-nucleolus organizer region (anti-NOR-90) specificity was reported to occur in Spanish cases of SSc (286). The antibody was also reported in two children with RP (101). However, others have estimated that less than 1% of SSc patients have anti-NOR-90 antibodies, and have reported that the antibody is not at all disease-specific, being found in a small proportion of SS, RA and SLE patients (76,103).

Other anti-nucleolar antibody specificities

Antibodies recognizing the nucleolar phosphoprotein B23 have been found to occur in more than 50% of GVHD, and in some SLE patients (360,366).

Predictive value of autoantibodies in Raynaud's phenomenon

Several different groups have detected autoantibodies of defined specificity in sera from primary RP patients who did not have a definite CTD diagnosis (171,357). These have included anti-topo I, anti-centromere, anti-Ro and anti-U1 RNP antibodies.

The significance of ANAs detected in 53% of 138 patients referred on the basis of severe RP was studied by Kallenberg *et al.* (170), by following the patients up over a six-year period (171). The patients were divided into primary RP (28%), secondary RP (55%), and suspected secondary RP (17%), on the basis of other CTD symptoms.

Initially, it had been found that ANAs (detected by IF methods) were more frequently found in patients with suspected secondary RP (71%), and in those with a defined CTD (66%), than in patients with apparently primary RP (who had no signs of a CTD) (16%) (170). Anti-topo I antibodies and ACAs were subsequently detected in the initial study sera by immunoblotting (IB). While 14/50 sera from SSc patients had ACAs, these were also detected in sera from 7/62 patients without a specific CTD diagnosis. At follow-up, three of these primary RP patients had since received a diagnosis of SSc. Further, two primary RP patients who had had topo I-positive sera had also developed symptoms fulfilling a diagnosis of SSc, and anti-Ro/La antibodies were detected in two sera from RP patients who went on to a diagnosis of SLE (171).

A prospective study of 77 primary RP patients without signs of CTD, carried out by Weiner *et al.* (358), showed essentially analogous results: a 63-fold risk of going on to develop signs of a CTD was calculated for those patients who tested positive for either anti-topo I or ACAs at the start of the study. Also, the presence of one of these autoantibodies was shown to be a more sensitive indicator of impending CTD symptoms than were results from widefield nailfold capillaroscopy. In the same study, ELISA methods were used to detect sera containing anti-topo I antibodies and ACAs. Two anti-topo I-positive, and two ACA-positive sera came from four patients who went on to develop tight skin at follow-up, three of whom subsequently attained a full diagnosis of SSc.

Together, these results illustrate the usefulness of autoantibody screening tests for patients with primary RP, in helping to identify those patients at risk of developing a defined CTD, and in predicting the particular CTD concerned. It is possible that ANoAs will also be shown to be useful prognostic markers in a minority of RP patients. Most interesting is the concept that antibodies may precede the full development of the symptoms with which they have found to be associated, thus placing autoantibodies at an early stage of aetiopathogenesis.

Significance of the autoantigens recognized by SSc sera

The cellular location and function of many autoantigens have now been determined. Consequently, associations between the biochemical roles of different autoantigens have emerged. Stefano (329) suggested that La autoantigen is a termination factor for RNAP III by demonstrating preferential binding of La to the RNAP III transcription termination signal. All rRNAs are transcribed by RNAP III, and the 60-kDa Ro particle may be involved in a discard pathway for defective 5s rRNA precursors (248). Nascent RNAP II transcripts have been found to occur in association with small nuclear ribonucleoproteins, including the U1 RNP particle (220). The Ku autoantigen was shown to be the regulatory component of the kinase complex responsible for phosphorylating RNAP IIA to form RNAP IIO (80). Finally, there is the recent identification of topo I as a transcription factor for RNAP II-transcribed genes (180).

Thus, various combinations of autoantigen which come together at certain stages of the cell cycle are recognized by sera of patients with autoimmune CTDs (125). It is thought that reactivity with one epitope on a complex may spread to other epitopes on the same particle(89). Antibodies to certain proteins such as centromere proteins, U3 RNP, Th RNP and Pm-Scl seem to be mutually exclusive in SSc, and there is no evidence for their direct interaction *in vivo.* However, it does appear that autoantigens which colocalize to the nucleolus are preferred targets for autoantibodies in SSc.

1.9 FAMILY STUDIES

As reviewed earlier, many genetic and environmental factors have been reported to cause or promote the development of SSc. Current thinking regarding aetiology, suggests a combination of genetic and environmental influences may be the best explanation of the available data, with an environmental trigger leading to disease in genetically susceptible individuals (138,331). However, the many possible ways in which individual factors may interact are complex and little understood. Indeed, one leading epidemiologist has described SSc as an epidemiological and genetic enigma (314). Moreover, even in the presence of the appropriate genetic and/or environmental stimuli, still other genetic polymorphisms may influence the particular subtype or severity of disease expression – as mentioned earlier, for instance, RP may represent a *formes frustes* of SSc (117). In order to track down the most significant genetic and/or environmental influences in SSc, to understand the interplay of such factors as the disease progresses, and to construct a general aetiological model (or models), further demographic research is vital, and this should include family studies.

The value of family studies

A familial background of immunological abnormalities is thought to be a contributory factor to the expression of SSc, and certain clinical features of CTDs have also been reported in the relatives of SSc patients. Such characteristics of SSc, occurring in an incompletely expressed form, can be assessed in family members of SSc patients by detailed and careful clinical examination, history-taking and laboratory investigations. Thus, in family studies, the characteristics of affected and unaffected individuals are inventoried in a setting where genetic and environmental influences have converged, in one or more individuals, to promote disease development and/or a set of features typical of the disease.

Shared genetic background

The first-degree relatives of large kinships provide a pool of individuals sharing many of the same genetic characteristics. Identical twins, where either one or both individuals are affected by the disease, would be of particular interest.

Shared environmental factors

Shared environmental influences are a further common feature of family members, particularly in the case of siblings who have lived together since early childhood. Another important group to consider is the spouses of SSc patients, who may have been exposed to similar environmental conditions in adulthood, whilst having very different genetic influences.

Genetic and environmental interactions

Multicase families are of particular value for the study of genetic and environmental interactions: certain differences between the genetic and/or environmental factors to which various members of the same family have been exposed may be found to coincide with affected versus unaffected individuals. Furthermore, by detecting more subtle clinical and immunological abnormalities, such information may also be obtained from the family members of isolated SSc cases.

Timing of disease onset

Genetic and environmental factors may be distinguishable according to patterns of disease onset in genetically related individuals (56,331). A strong genetic component might tend towards a non-contemporaneous onset of disease for individuals of different generations, while environmental influences may be reflected by simultaneous disease onset in individuals of different ages. Study of multicase families, where individuals from different generations are found to be affected by SSc, would be of particular significance in this regard.

Multicase family studies

Systemic sclerosis is a very rare disease: multicase SSc families are extremely unusual. However, some clinical and immunological features of the disease have been reported in first-degree relatives of patients. Most of this information has come from case studies of individual affected kindreds, and the genetic and environmental background of such families is sometimes difficult to assess. A variety of relationships have existed between the family members in these reports, e.g. mother/daughter (61,99,241), mother/son (117), sister/sister (61,138), sister/brother (138,310), brother/brother (310), and aunt/nephew (334).

Multicase systemic sclerosis

One early report by Frayha *et al.* described a mother-daughter pair, each having lc-SSc together with SS. Both patients had the same autoantibody specificity, and developed their first symptom, RP, within one year of each other (99). Other multicase families have since been reported (61,71,117,138,223,235,241,295,310,334), with the following general findings: (i) in several such instances, there have been considerable similarities in disease expression between the related SSc patients (241,334); (ii) as recorded in a review by Christy & Rodnan (56), it was found that, of eleven cases surveyed, eight

recorded the interval between the onset of the disease in the two affected individuals as being three years or less; (iii) the same HLA alleles have often been detected in the involved family members (61,138,223,235,241,310); (iv) where an environmental agent was suspected in a particular case, both affected family members had been exposed to it (56,295). Some of these findings are exemplified below.

One particularly interesting paper described five siblings: two of the brothers and a sister had SSc, and a brother and a sister had RP (310). Two other close relatives had features of a CTD. The two brothers with SSc and their sister with RP had an identical HLA haplotype (A2, B21, DRw4/A2, B12, DRw4).

Of especial note was a report of conjugal SSc (56). This husband and wife, who had lived together for 31 years before onset, both developed the disease within three years of each other, and, in fact, both had HLA-A1, -B8 and -DR3 alleles. The husband was a stonemason/cement mixer, with considerable silica exposure. Following detailed history-taking it emerged that the wife had hand-washed all her husband's overalls, which were likely to be contaminated by silica dust.

A study by Hietarinta (138) included a very important finding: in a family with three cases of SSc (two sisters and a brother), it was found that all three had the HLA alleles A2, B8, DR3, as did another sibling with immunological abnormalities. However, a further four healthy family members also had these alleles. Furthermore, three of them (all siblings of the SSc patients) were reported to be HLA identical with two of the patients (HLA-A, -B, -C and -DR alleles were assessed in this study). It was concluded that the A2, B8, DR3 haplotype is a predisposing factor for SSc, but that other genetic and/or environmental influences are required for disease expression.

The interplay of genetic and environmental factors was apparent in the paper of Sanchez-Roman *et al.* (295). In their prospective study of workers exposed to silica dust at a scouring powder factory, they found that several sets of siblings had worked there together, and that, in two families, more than one had developed SSc.

Multicase connective tissue disease

Reports of multicase SLE families are also to be found (57,295). In some cases, both SSc and SLE have been found in different members of the same kindred: Flores *et al.* recorded a high degree of serological and HLA concordance in eight such cases studied (95). Such findings suggest some commonality in the aetiology of these two disorders, and it was suggested by the authors that these factors were genetic rather than environmental, since three of the pairs had never lived together.

Meanwhile Molta *et al.* reported on the families of two unrelated SSc patients in which multiple cases of CTDs occurred, including discoid lupus, SS, RP, RA and psoriasis and ulcerative colitis (235). A similar family was described by Sels *et al.*, with cases of RA, SLE, psoriasis and inflammatory bowel disease were found (306). Familial aggregation of RP has also been reported (100).

Immunologically based family studies

Autoantibodies in systemic sclerosis family members

Immunological abnormalities in the relatives of SSc patients were first noted in 1961 by Corcos *et al.* (64). This group reported that 37% of 78 blood-relatives of SSc patients had abnormal IgG levels. A number of other papers have been published since then, and the following points have been made with a fair degree of consistency: (i) an increased incidence of anti-nuclear reactivity has been reported in sera from the consanguineous relatives of SSc patients; (ii) a less pronounced increase in anti-nuclear reactivity in sera from spouses of SSc patients has also been reported; (iii) SSc-specific antibodies are only present in patients with the disease, or in certain cases, in individuals who are in suspected transition to SSc; (iv) in multicase SSc families, the affected individuals have tended to express the same autoantibody specificity. A variety of examples is presented below.

An early study of 90 relatives of SSc patients by indirect immunofluorescence (IF) observed that, although CTDs were not common in the relatives, ANAs were detected in some 58%, compared with only 2% of normal controls (90). Some time later this finding was confirmed by Rothfield *et al.* (289), who detected ANAs in 12% of 83 first-degree relatives of SSc patients, in one of 17 spouses (6%), and in only 4% of 50 healthy controls (289). In this study, it was also noted that, while 10% of ANA-positive patients had high-titre ANAs, all ANA-positive blood-relatives had low-titre antibodies.

A particularly thorough serological study of 21 SSc patients and their 35 first-degree relatives was carried out by Takehara *et al.*, using IF and immunodiffusion techniques (336). None of the family members had a defined CTD. The use of the new HEp-2 cell-line in this study showed that 100% of patients had positive IF-ANAs (1:40 serum dilution), while the rate in relatives was 26%, and, of twenty control normals, only one was positive (5%). Of the relatives, several were found to have high-titre IF-ANA (one 1:2560, one 1:1280, three 1:640), while the normal control was positive at 1:160 dilution. It was also noted that positive ANAs were more common in daughters of patients than in sons (54% versus 7%, respectively). Patterns detected included fine nuclear speckles and granular patterns. Of note was the detection of ACAs (by IF) in the mother of an anti-topo I-positive SSc patient. Interestingly, the titre of this serum was 1:320, which would be considered unusually low in SSc. Interesting findings by immunodiffusion included anti-U1 RNP antibodies in the brother and in the daughter of one SSc patient who herself had anti-topo I/U1 RNP antibodies. The overall rate of positivity by immunodiffusion was thus 6% of SSc relatives (2/35), as compared with 76% of patients and 0% of normal controls.

A subsequent study by Ruffatti *et al.* specifically concerned the incidence of ACAs and ANAs in the 116 blood-relatives of 22 ACA-positive SSc patients (292). A total of 82 healthy normal controls were included. Scleroderma was absent from relatives, though some features of RP were present in eight of them. A low-titre ACA pattern

(1:40) was found by IF in four relatives (3%) and in none of the controls. Of the four ACA-positive relatives, one had hypoparathyroidism, one had myasthenia gravis, and one had cold-sensitivity. When ANA-IF was examined, 17 (15%) of relatives were found to be positive (one had a titre of 1:320), and all of these were first-degree relatives. Of the normal controls, only four (5%) were positive (all titres were 1:40 or 1:160), a result which was significantly different from the relatives. Positive immunodiffusion results were obtained for one proband (topo I), no relatives and no controls.

Importantly, not all investigators have supported these findings. The study of Barnett & McNeilage in 1993 produced no evidence that environmental or genetic factors influence the development of ANA positivity in SSc. Their paper describes a study of 58 SSc patients, their 30 spouses and 74 of their first-degree relatives, together with 66 age-matched controls (13). They found positive ANA (titre 1:40 or above) in 95% of patients, 7% relatives, 3% spouses and 18% of controls. No defined ANAs were detected in family members or normals.

Autoantibodies in connective tissue disease family members

A higher than normal incidence of ANAs has also been noted in family members of SLE patients (74,289). In the latter study, a key finding was that close household contact with the proband was associated with ANA positivity. Furthermore, a study of multicase SLE families by Cleland *et al.* detected ANAs in 53% of consanguineous and 57% of non-consanguineous relatives, as compared with 5% of controls (57).

Southwood *et al.* conducted a study of ANAs in juvenile chronic arthritis patients and their families, and found that 70% of patients and 17% of relatives had IF-ANA. Furthermore, in five families, IB studies showed common banding patterns between the probands and other family members. It was suggested that there is an inherited component to the autoimmune response in some of these families (322).

Other abnormalities in the family members of systemic sclerosis patients

Chromosome instability

An increased level of chromosome instability compared with controls has been reported in SSc patients and in their family members (8). This was implied by a significant increase in the number of alterations occurring to the variable number tandem repeat sequences (minisatellites). A further report by the same group found that telomeres were also significantly shorter in SSc patients, as well as in their spouses and blood-relatives. It was suggested that chromosome instability may be due to an environmentally related cause (8,9). However, a report by another group found no evidence of actual chromosome damage in SSc patients or their relatives (31).

Complement deficiency

It was reported that 67% of SSc patients, 49% of SSc blood relatives, 33% of spouses and only 17% of normal controls had a C4 null allele (264). A significant association between the presence of a C4 null allele and/or a DR5 allele and the presence of an ANA was observed for both patients and relatives.

Endothelial-related abnormalities

Compared with controls, significantly raised levels of a Factor VIII-related antigen have been detected in 20% of the first-degree relatives of SSc patients and in 15% of their spouses (159). This compared with raised levels in 62% of SSc patients and 17% of primary RP patients. Furthermore, one of two spouses with a positive result had RP and was ANA-positive. In the case of the SSc patients, those with either ACAs or anti-U1 RNP antibodies had a significantly higher rate of positivity than the remaining patients. These results are indicative of an abnormal degree of EC damage occurring in these individuals. Furthermore, the data suggested that EC damage and the propensity for autoantibody production in the relatives are related in some way (159). These results were especially significant in light of earlier findings by Sheldon *et al.* (310). In their report, five siblings were described, three of whom had SSc, with a further two having RP. Endothelial cell cytotoxicity assays revealed positive results in 2/3 of the SSc patients, both RP patients and in a further 3/13 healthy relatives.

Twin studies

Information concerning monozygotic twins, one or both of whom are affected by SSc, is clearly of paramount significance. However, it should be remembered that twins are not necessarily identical. Such differences have been highlighted by Gregersen *et al.* (118), and include X-chromosome inactivation, placental separation, somatic development and stochastic events.

A pair of concordant identical twins with SSc was reported by Cook *et al.* (61). A review of their genetic and environmental background suggested that genetic factors were of greatest importance in this particular case, particularly as their mother also had SSc (61).

Abnormal lymphocyte function in a pair of discordant monozygotic twins was investigated by Dustoor *et al.* (79). Abnormalities were detected in the SSc-affected twin (e.g. low absolute T cell count; high $CD8^+$-T cell count; decreased mitogen responsiveness; increased Th-cell help for Ig synthesis) but not in the healthy twin, and the authors concluded that the immunological defects in scleroderma are not completely genetically determined.

An interesting study of discordant and concordant monozygotic twins with regard to MS were studied by Utz *et al.* (348). The myelin basic protein (i.e. the self-antigen implicated in MS) and the tetanus toxoid antigen were separately incubated with peripheral blood lymphocyte cultures. It was found that TCR usage by control twins

and by concordant twins involved the selection of similar Vα chains by the related twins, and this was true in the case of either stimulating antigen. However, related discordant twins selected different Vα chains in the case of both stimulating antigens. The preference for certain Vα chains was antigen-specific. It was postulated that environmental factors or somatic events were responsible for skewing the TCR repertoire in MS. The authors suggested that either superantigens, or a chronic T-cell mediated disease may be responsible.

1.10 AIMS OF THIS STUDY

In order to explore in more detail the relative importance of genetic and environmental factors in the expression of SSc autoantibodies, the clinical, serological and immunogenetic features of sixty-five SSc patients, together with their relatives and spouses, were studied by the U.K. Systemic Sclerosis Study Group (206), with a follow-up study being carried out seven years later (207,226,331,361).

One report by members of the group specifically considered four multicase SSc families (331). Disease subtype assignation was the same for each pair of related patients, and three of the four pairs had common HLA-DR alleles. The clinical features of the related SSc patients were reported to be similar in the mother/daughter pair, in the father/daughter pair, in the sister/sister pair, as well as in the identical twin pair. The onset of disease for each pair was considered with regard to both absolute dates as well as with respect to each individual's age. Based on these results, it was concluded that environmental factors were involved in the appearance of SSc in these multicase families (331).

Other interesting information was provided by the SSc family study. For example, relatives with other CTDs occurred, and included RP, RA, SLE, Pm, morphea and pernicious anaemia (PA). Probands had a significantly higher incidence of HLA-DR3, -DR5 and C4A null alleles compared with controls. Anti-topo I and ACAs were found exclusively in probands or family members with SSc and not in unaffected relatives, and were closely associated with certain HLA-DQB1 alleles (226,361). Furthermore, ANAs characteristic of other CTDs, such as SLE, were also found to be limited to individuals with a defined CTD (206,207). In the present study, the frequency and type of ANoAs have been investigated in the same families.

While the distribution and disease subtype associations of the SSc-specific ANAs, anti-topo I and ACAs, have been well studied, much less data is available concerning ANoAs, which are also very common in SSc. As already mentioned, all antigens known to be recognized by SSc-specific antibodies have been found to co-localize to the nucleolus at some stage of the cell cycle. Given that the nucleolus appears to be the focal point of autoimmune responses in SSc, the importance of detecting anti-nucleolar reactivity in family members of SSc patients can be appreciated. Such work may lead to

a greater understanding of aetiology and pathogenesis. Therefore, using the highly sensitive technique of radio-immunoprecipitation (IP), the present study aimed to identify the antinucleolar reactivities occurring in serum samples taken from these family members. Since the method of IP is extremely sensitive, the detection of additional antinuclear specificities not found in the original study was also anticipated. The technique involves the radiolabelling of proteins including RNPs expressed by human cell lines, followed by incubation of the radiolabelled cell extracts with Protein-A Sepharose CL-4B beads precoated with patient antibodies. The proteins and RNPs bound to the beads are then extracted, separated on polyacrylamide gels, and detected by autoradiography (proteins) and silver staining (RNAs). Other techniques, such as IF, IB and Ouchterlony double immunodiffusion were also used, with the aim of complete autoantibody profiling of each serum.

Summary of initial aims and objectives

The initial aims and objectives of the present study were as follows:

(i) To determine the prevalence of IF-ANAs and IF-ANoAs in the sera of SSc patients and their family members

(ii) To assess the prevalence of previously characterized ANAs and ANoAs in the sera of SSc patients and their relatives by IP, and to further define their clinical associations

(iii) To report and identify any novel antinuclear or antinucleolar antigens recognized by the study sera

(iv) To further investigate any unusual autoimmune features in the family members of SSc patients

Expected value of results

The study as a whole was intended to provide further information about genetic and environmental factors operating in the expression of disease-specific autoantibodies, which may contribute to an understanding of the pathogenesis and aetiology of the disease. It was also hoped that a more detailed examination of the relationships between ANoAs and clinical expression of the disease would provide clinicians with additional information helpful in the diagnosis and prognosis of individual patients.

CHAPTER 2

MATERIALS AND METHODS

2.1 PATIENTS AND SERA

2.1.1 Selection of patients, relatives and controls

For the SSc family study (Chapter 3), 62 SSc patients were selected, and 239 of their relatives agreed to participate. One hundred-and-twenty normal control samples were also obtained, taken from a bank of sera from healthy donors.

For the subsequent study of anti-RNAP antibodies and their clinical associations (Chapter 4), the 62 SSc patients from the family study were included, together with nine patients from a Si-SSc study (227), and 144 additional consecutive SSc patients recruited from the Royal National Hospital for Rheumatic Diseases, Bath, U.K. (RNHRD).

2.1.2 Diagnostic criteria and scoring systems

Clinical examination

For the SSc family study, clinical assessments of probands and their relatives were carried out by clinicians at the various participating centres (Dr N.J.McHugh and Professor P.J.Maddison, RNHRD; Professor C.M.Black, Royal Free Hospital, London; Dr R.Bernstein, Royal Infirmary, University of Manchester; Dr P.Bacon, Rheumatism Research Wing, University of Birmingham; Dr M.I.V.Jayson, Hope Hospital, Saltford; Dr N.G.Gusseva, Russian Academy of Medical Sciences, Moscow, U.S.S.R.; Dr N.J.Olsen, Vanderbilt University, Nashville, TN, U.S.A.). Clinical field work (U.S.S.R. and U.S.A.) was also undertaken by Dr C.Stephens (Royal Free Hospital).

For the study of anti-RNAP antibodies, additional SSc patients were examined by Dr N.J. McHugh and Dr Y. Patel at the RNHRD, while Si-SSc patients were assessed by Dr U.F.Haustein, University of Leipzig, Germany.

Diagnostic criteria and disease subtype classification

In 1980 the ARA published preliminary criteria for the diagnosis of SSc (218). This document has since become the accepted world-wide standard for research purposes. The major criterion is scleroderma proximal to the metacarpophalangeal joints, while minor criteria are (i) sclerodactyly, (ii) digital pitting scars of the fingertips, or loss of substance of the distal finger pad, and (iii) bibasilar pulmonary interstitial fibrosis. Either the main criterion or at least two minor criteria must be met for a diagnosis of SSc (97% sensitivity and 98% specificity (218)).

Raynaud's phenomenon

In the SSc family study, the presence of RP in the family members of SSc patients was recorded, being defined as a biphasic or triphasic response to cold (16,218).

Disease subtype assignation

In both the SSc family study and the anti-RNAP antibody study, disease subtype was classified as either lc-SSc or dc-SSc (198). Limited cutaneous SSc was defined as cutaneous sclerosis not extending proximal to the elbows or knees, with or without

facial involvement, while dc-SSc was defined as cutaneous sclerosis extending proximal to the elbows or knees.

Scoring systems

Clinical data were recorded in greater detail for the anti-RNAP antibody study:

Disease subtype classification

Where possible, a modified version of Barnett's classification system (13,225) was used to assign each patient to one of three categories based on the extent of cutaneous involvement: (1) sclerodactyly only, (2) skin involvement limited to the hands, forearms, face and neck, or (3) diffuse skin involvement. In practise, this meant a further subdivision of the lc-SSc patients (as defined above) into either category 1 or 2, while patients with dc-SSc (as defined above) were recorded as being in category 3.

Pulmonary involvement

Pulmonary involvement was assessed by chest X-ray and standard DLCO tests. If the DLCO value was <80% of that predicted, and/or significant shadowing of the lungs characteristic of bibasilar pulmonary fibrosis was apparent by X-ray examination, pulmonary involvement was recorded (225). Further classification into mild, moderate or severe involvement (where possible) was according to the definitions described in Appendix I.

Renal involvement

One or more of the following features (unless attributable to unrelated disease) was taken to imply renal involvement: (i) creatinine clearance rate <60 ml/min (or serum creatinine >1.3 mg/dl), (ii) active urinary sediment, (iii) proteinuria (>0.5 g/day), and (iv) accelerated hypertension (diastolic B.P. >105 mmHg) (225). Again, severity classifications were recorded where possible, as described in Appendix I.

2.1.3 Preparation and storage of sera

Samples for the family study, and all Si-SSc patient samples, were supplied as frozen sera for immediate storage at -20°C. Samples from the RNHRD were supplied as freshly collected whole blood in unheparinized 10-ml collection tubes, from which serum was prepared by the following method.

Method 2.1.3: Extraction of serum from human blood

Following collection, the blood-sample tubes were gently mixed, and left at room temperature for at least 30 min. After clotting had occurred, the tubes were centrifuged at room temperature in a Hereaus Labofuge 6000 Centrifuge (Hereaus Instruments) for 10 min at 3000 r.p.m. (Centrifugation Protocol 1: see Appendix II). Serum supernatants were removed, with several aliquots (0.5-2.0 ml) of each sample being stored at -20°C. Excessive freeze-thawing of sera was avoided during subsequent investigations.

2.2 TISSUE CULTURE TECHNIQUES

Tissue culture (TC) procedures were performed in standard Class II TC cabinets with lamina flow of high-pressure filtered air. Ethanol-sprayed gloves were worn, and all reasonable care was taken to avoid extraneous infection of cultures. All solutions and growth media were prepared using double-distilled water, which was passed through a Milli-Q water purification system (Millipore, Watford, U.K.), and autoclaved in-house prior to use. Pasteur pipettes, microfuge tubes, TC bottles and pipette tips were also autoclaved.

Unless stated otherwise, sterile TC-media concentrates and media-supplementing solutions were obtained from Life Technologies Ltd, Paisley, U.K.; sterile TC-grade plastics (Falcon brand) were from Becton Dickinson Labware, New Jersey, U.S.A., and general laboratory reagents were obtained from Sigma Chemical Co. Ltd, Poole, U.K.

2.2.1 Preparation of tissue-culture media

Method 2.2.1a: Preparation of RPMI media

Rosewell Park Memorial Institute 1640 medium (RPMI) was obtained as a 10x concentrate without sodium bicarbonate or glutamine. A 50-ml volume was diluted in 375 ml water, and supplemented with 15 ml sodium bicarbonate solution (7.5% w/v), 5 ml penicillin/streptomycin solution (10000 I.U./ml and 10000 µg/ml respectively), and 5 ml L-Glutamine solution (200 mM). The volume was made up to 500 ml with water ('Serum-free RPMI'), or with 50 ml heat-inactivated foetal calf serum (FCS) ('RPMI+10% FCS'). The pH of the medium was adjusted to pH7.0-7.5 by adding drops of saturated sodium hydroxide solution (25 ml water were equilibrated with 10 g sodium hydroxide pellets and sterilized by passing through a small bottle-top filter (pore size 0.22 µm; Millipore) from a solution-filled syringe).

Method 2.2.1b: Preparation of methionine-deficient RPMI media

A powdered RPMI base, deficient in sodium bicarbonate, glutamine, methionine, leucine and lysine, was obtained from Sigma Chemical Co. Ltd. One container of the non-sterile powder supplied was dissolved in 775 ml water, as instructed by the manufacturer. To sterilize, the solution was passed through a large bottle-top filter (cellulose acetate membrane, 0.22 µm pore size; Becton Dickinson Labware) attached to a vacuum pump. The medium was then supplemented with 30 ml sodium bicarbonate solution (7.5% w/v), 10 ml penicillin/streptomycin solution (10000 I.U./ml and 10000 µg/ml respectively), 10 ml L-Glutamine solution (200 mM), 5 ml L-Leucine (200x solution for TC; Sigma Chemical Co. Ltd) and 5 ml L-lysine (200x; Sigma Chemical Co. Ltd). The volume was made up to 1 l with water ('Serum-free Met-deficient RPMI'), or with 50 ml dialysed FCS (d-FCS; prepared according to Method 2.2.1*c*)

('Met-deficient RPMI+5% d-FCS'). The pH of the medium was adjusted to 7.0-7.5, if necessary, as described above.

Method 2.2.1c: Preparation of dialysed foetal calf serum

Beginning in the morning, an 800-mm length of dialysis tubing (20-mm in diameter; Sigma Chemical Co. Ltd) was washed according to the manufacturer's instructions and used immediately. After two tight knots had been made in one end of the tube, FCS was poured into the other end until the tubing was about 3/4 full. The open end was similarly secured, and the tubing was briefly rinsed in phosphate-buffered saline (PBS, prepared from PBS tablets according to manufacturer's instructions (Oxoid Unipath Ltd, Basingstoke, U.K.)) to remove any spillages. It was then put into a large beaker containing 3 l of ice-cold PBS, which was placed on a magnetic stirrer at 4°C for about 8 h. The tubing was placed in fresh PBS overnight, and again the following morning. After a total of ~30 h cold dialysis, the tubing was rinsed in PBS, cut open and carefully emptied.

Before adding to medium, it was necessary to filter-sterilize the d-FCS: a 10-ml syringe was filled, and attached to a small bottle-top filter (pore size 0.22 μm; Millipore), through which the serum was forced in a dropwise fashion.

2.2.2 Maintenance of cell lines

The following cell lines was maintained in standard TC incubators programmed to a temperature of 37°C, with 5%-carbon dioxide-enriched air.

Maintenance of the HEp-2 cell-line

The adherent HEp-2 cell-line is derived from a human orolaryngeal carcinoma. Cells were maintained in 100-mm-diameter petri dishes in ~10 ml RPMI+10% FCS. Fresh medium was applied 1-2 times per week, until plates were confluent (~2 million cells per plate): the cells were then washed and resuspended (Method 2.2.2*a*) for passaging (see below), storage (Method 2.2.2*b*) or preparation of HEp-2 slides (Method 2.2.3).

To passage cells, 50 μl of washed, resuspended cells were added to each new plate along with 10 ml fresh medium. The plates were gently swirled, and placed in the TC incubator: cells were then allowed to recover and adhere overnight.

Maintenance of the K562 cell-line

Derived from a human chronic myelogenous leukaemia (ECACC No. 89121407), these fast-growing, non-adherent cells were maintained in 75-cm^2 TC flasks in ~30 ml RPMI+10% FCS. Cells were checked every two or three days until flasks were confluent (~7-35 million cells/flask): the cells could then be split (see below), stored (Method 2.2.2*b*), or used immediately to prepare cell extracts (Section 2.2.4).

To split cells, 5 ml of confluent cell suspension was added to 25 ml fresh medium in a new flask. Alternatively, cells were rapidly expanded by splitting the confluent suspension between two new flasks, and topping up to 30 ml with fresh medium. When

particularly large quantities of cells were required, 175-cm^2 flasks were used, containing 100 ml of culture (20-100 million K562 cells per flask when confluent).

Method 2.2.2a: Resuspension of HEp-2 cells

At least ten plates of confluent HEp-2 cells were processed at one time. Aliquots of trypsin-EDTA (1x solution in modified Puck's Saline A; Life Technologies Ltd) were first thawed at room temperature. Medium was carefully removed from the plates by aspiration. Each plate was immediately washed with 10 ml PBS, after which 2.5 ml trypsin-EDTA was added. The plates were gently swirled, and incubated at room temperature for 2-5 min: during this time frequent progress checks were made under the microscope, and the plates were swirled occasionally. When the majority of cells had floated into suspension, trypsinization was halted by adding about 10 ml RPMI+10% FCS to each plate with gentle swirling, and the cell suspensions were pooled into an appropriate number of 50-ml centrifuge tubes. The suspensions were centrifuged in a Beckman GPR/GS-15R centrifuge set at 1°C for 5 min at 1500 r.p.m. (Centrifuge Protocol 2). After carefully pouring off supernatants, the cells were resuspended in fresh Serum-free RPMI medium (~2 ml per tube) by gently mixing up and down with a 1-ml pipette. Cells from different tubes were pooled into one centrifuge tube, and the volume was made up to 50 ml with Serum-free RPMI medium to wash. After recentrifuging according to Protocol 2, the supernatant was poured off, and the cell pellet was very slowly resuspended in a total of 2 ml Serum-free RPMI medium using a 0.5-mm bore needle (Becton Dickinson Labware) and syringe. The cells were then counted using a haemocytometer, after which an appropriate amount of Serum-free RPMI medium was mixed in to give a final concentration of 10 million cells/ml.

Method 2.2.2b: Freezing tissue-culture cells

Tissue-culture cells were frozen down in liquid nitrogen for long-term storage. A concentration of around 4-5 million cells/ml is usually recommended.

HEp-2 cells

About 25 confluent plates of HEp-2 cells were required for the preparation of one batch of ten 1-ml aliquots of HEp-2 cells (50 million cells in total). The appropriate volume of resuspended HEp-2 cells was first prepared, as described in Method 2.2.2*a*. An equal volume of freezing solution (20% dimethylsulphoxide : 50% FCS : 30% Serum-free RPMI) was gently mixed in (final concentration 5 million cells/ml), and 1-ml aliquots were placed in 2-ml screw-top freezing vials. The vials were securely tightened, wrapped in cotton wool, and placed in a polystyrene box, which was stored at -80°C overnight. Next morning the vials were transferred into a liquid nitrogen bank for long-term storage.

K562 cells

About 150-200 ml confluent K562-suspension was required for the preparation of twenty 1-ml aliquots of K562 cells (100 million cells in total). After counting the

pooled contents of several flasks, the appropriate volume of confluent culture was decanted into 50-ml centrifuge tubes, and centrifuged according to Protocol 2. After carefully pouring off supernatants, the cells were resuspended in Serum-free RPMI (~2 ml per tube) by gently mixing up and down with a 1-ml pipette. Cells from different tubes were pooled into one centrifuge tube, and the volume was made up to 50 ml with Serum-free RPMI to wash. After recentrifuging according to Protocol 2, the supernatant was poured off, and the cell pellet was very slowly resuspended in a total of 5 ml Serum-free RPMI using a 0.5-mm bore needle (Becton Dickinson Labware) and syringe. The cells were re-counted, after which an appropriate amount of Serum-free RPMI was mixed in to give a concentration of 10 million cells/ml. An equal volume of freezing solution was gently mixed in (final concentration 5 million cells/ml), and cells were prepared for freezing as described above for HEp-2 cells.

Method 2.2.2c: Thawing tissue-culture cells

A single vial of stored cells was removed from the liquid nitrogen store and immediately rinsed, with gentle shaking, under very hot, running tap-water. Once thawed, the outside of the vial was dried with tissue paper, and swabbed with ethanol. The contents of the vial were then added to a 25-ml centrifuge tube containing 10 ml RPMI + 10% FCS, and gently mixed in. The cells were centrifuged according to Protocol 2 after which the supernatant was poured off, and the pellet resuspended in 2 ml RPMI + 10% FCS using a 1-ml pipette. The volume was made up to 10 ml and the resuspended cells were added to a 10-mm petri dish (HEp-2 cells) or to a 25-cm^2 flask (K562 cells), and allowed to recover overnight.

2.2.3 Preparation of HEp-2-cell slides

Method 2.2.3: Preparation of HEp-2-cell slides for immunofluorescence

Forty 10-well, glass, multispot microscope slides (C.A.Hendley (Essex) Ltd, Loughton, U.K.) were cleaned with ethanol and a soft cloth. After air-drying, slides were double-bagged, autoclaved, and stored, ready for use.

To prepare each batch of forty HEp-2 slides, ten plates of HEp-2 cells were grown to confluence (Section 2.2.2). Cells were trypsinized, washed, counted, and resuspended in a total of 20 ml RPMI + 10% FCS (1 million cells/ml), according to Method 2.2.2*a*.

Slides were laid out inside 100 x 100-mm square petri dishes (four slides per plate) to maintain sterility. A 50-µl drop of the diluted cell suspension was then placed on each well of the microscope slides, taking care that the medium covered the whole surface of each well (a pipette tip was used to guide the medium where necessary). The petri dishes were placed in TC incubators overnight.

Next morning slides were individually rinsed twice by pouring PBS over them. The slides were placed in a slide rack and immediately dunked in an ice-cold (-20°C) acetone bath for a few seconds. Next they were placed in another ice-cold acetone bath

for 5 min to fix the cells. Fixed slides were laid out to dry for 5 min, after which they were wrapped in aluminium foil, and stored at -20°C, ready for use.

2.2.4 Preparation of K562-cell extracts

Method 2.2.4a: Preparation of ^{35}S-methionine-radiolabelled K562-cell extract

Radiolabelling

Radiolabelling was based on the method of Craft, Mimori & Olsen (1988) (66). About 400 ml of semi-confluent, actively proliferating, K562-cell culture suspension were used for the preparation of one batch of radiolabelled K562-cell extract (~80 million cells prior to labelling). After counting, the appropriate volume of cell suspension was decanted into 50-ml centrifuge tubes, and centrifuged according to Protocol 2. Each pellet was resuspended in about 2 ml Serum-free Met-deficient RPMI using a 1-ml pipette, and cells were pooled into two 50-ml centrifuge tubes. Each of the two tubes was made up to 50 ml with Serum-free Met-deficient RPMI, and gently inverted to wash the cells. After further centrifugation as before, each washed pellet was resuspended in 2 ml Met-deficient RPMI + 5% dFCS, and cells were pooled; the total volume was made up to 50 ml with Met-deficient RPMI + 5% dFCS, and cells were re-counted using a haemocytometer.

An appropriate volume of the cell suspension was added to each of four 175-cm^2 TC flasks (20 million cells/flask), and the volume of each flask was made up to 80 ml (density 0.20-0.25 x 10^6 cells/ml was acceptable (66)). The suspension was gently swirled to mix, and the flasks were put in the TC incubator for about 20 min, in order to deplete the intracellular pool of unlabelled methionine. The addition of 160 µl ^{35}S-labelled L-methionine solution (6.7 µM; 10 mCi/ml; NEN Biologicals, NEN Dupont, Stevenage, U.K.) to each of the four flasks gave a final activity of 20 µCi/ml. The flasks were gently swirled, and returned to the incubator for overnight labelling (16-18 h: half the doubling time of the cell line (66)).

Preparation of cell extract

The following morning the condition of the cells was checked under the microscope: if a large proportion of cells was judged to be dead the procedure was abandoned. The suspension was transferred into 50-ml centrifuge tubes, and the cells were centrifuged according to Protocol 2. From this point on, all work was done on ice. After pouring off the supernatants, each pellet was resuspended in 5 ml ice-cold tris-buffered saline (TBS: 10 mM Tris.Cl pH7.4, 150 mM NaCl), and all cells were pooled into a 50-ml centrifuge tube. The volume was made up to 50 ml with TBS, and gently inverted to wash the cells. After further centrifugation as before, the cell pellet was resuspended in a total of about 8 ml protein IP buffer (IPP: 10 mM Tris.Cl pH8.0, 500 mM NaCl, 0.1% (v/v) Nonidet P-40 (NP-40)), which caused cell lysis. Lysed cells were sonicated in the 50-ml centrifuge tube using a sonicator (Soniprep 150 MSE) at setting 3 (three periods of 60 sec separated by 20-sec rests).

Immediately following sonication the cell extract was transferred to a number of 1.7-ml microcentrifuge tubes, which were centrifuged in a Beckman GS-15R/F2402 at a speed of 12 000 r.p.m. for 20 min at 1°C (Centrifuge Protocol 3). Following centrifugation, the supernatants of cell extract were removed from the tiny pellets of insoluble cell debris, transferred into clean microfuge tubes in 1.25-ml aliquots (total volume approximately 7.5 ml), and stored at -80°C for later use. Each aliquot of final K562-cell extract corresponded to ~12.5 million cells before labelling (cells were not counted after overnight labelling for safety reasons).

Method 2.2.4b: Preparation of unlabelled K562-cell extract for IP

With the exception of the overnight labelling step, an adapted Method 2.2.4*a* was used to prepare unlabelled K562-cell extracts suitable for affinity purification of autoantigens by IP (see Method 2.3.3*d*).

About 200-300 ml of confluent K562-cell culture suspension was used for the immediate preparation of one batch of unlabelled K562-cell extract (150 million cells: total yield of cell extract ~7.5 ml, stored in 1.25-ml aliquots at -80°C).

Method 2.2.4c: Preparation of unlabelled K562-cell extract for RNA-IP

For the preparation of K562-cell extracts suitable for RNA-IP experiments (Method 2.3.3*b*), RNAse-free conditions were maintained using standard precautions (see Appendix 2). Otherwise, the same basic method as described above (Method 2.2.4*a*) was used: modifications are detailed below. The method was an adaptation of that described by Forman, Nakamura, Mimori *et al.* (1985) (96).

About 200-300 ml of confluent K562-cell culture suspension were used for the preparation of one batch of extract (150 million cells). After washing the cells in TBS as described in Method 2.2.4*a*, the cell pellet was resuspended in a total of about 8 ml of an adapted (high Tris, low salt) IP buffer (NET-2: 50 mM Tris.Cl pH7.4, 150 mM NaCl, 0.05% (v/v) Nonidet P-40). Lysed cells were then sonicated and centrifuged, as described in Method 2.2.4*a* (total yield of cell extract ~7.5 ml, stored in 1.25-ml aliquots at -80°C, or used immediately).

Method 2.2.4d: Preparation of unlabelled K562-cell extract for immunoaffinity column chromatography

The same basic method as described above (Method 2.2.4*a*) was used to prepare unlabelled K562-cell extracts suitable for purification of autoantigens by immunoaffinity column chromatography (Section 2.3.4). Modifications are detailed below (adapted from a combination of the methods of Craft *et al.* (1988) (66), Plunkett & Springer (1986) (269), and Springer (1993) (324)).

About 900-1100 ml of confluent K562-cell culture suspension were used for the preparation of one batch of extract (500 million cells). After washing the cells in 100 ml TBS, as described in Method 2.2.4*a*, the cell pellets were pooled and resuspended in a

total of 5.5 ml modified (low salt) IP buffer (MIP: 10 mM Tris.Cl; 140 mM NaCl; 0.1% (v/v) NP-40). Lysed cells were then sonicated and centrifuged as described in Method 2.2.4*a* (total yield of cell extract ~5.0 ml, stored at -80°C or used immediately).

2.3 SEROLOGICAL AND BIOCHEMICAL TECHNIQUES

2.3.1 Ouchterlony double immunodiffusion

Technical basis

An antigen-rich saline-extracted tissue suspension and a test serum are put into separate, adjacent wells of a horizontal agarose gel. The porous gel allows diffusion of both antibodies and soluble antigens: should the serum sample contain antibodies which recognize components of the extract, a visible precipitin line will form between the two wells, consisting of an insoluble immune complex ('zone of equivalence'; Fig. 2.1*a*). It is important that sufficient quantities of antigen and antibody are present, and that the respective wells are at an appropriate distance apart, otherwise a false negative reading may result.

Once reactivity of a serum with a component of a particular extract has been demonstrated, the serum is re-tested, this time placing a prototype serum of known antibody specificity in an equivalent well close by (Fig. 2.1*b* and *c*). Examination of the physical interaction between two adjacent precipitin lines indicates whether the two sera contain antibodies which recognize the same, different, or related antigenic particles (Fig. 2.1*d*).

Important advantages of the Ouchterlony technique in terms of the present study are (i) its ability to discriminate between anti-U1 RNP sera (i.e. sera recognizing only U1 RNP-specific subunits) and sera recognizing both U1 RNP-specific and Sm subunits (the Sm subunits being found on other UsnRNPs as well as on U1 RNP); (ii) its ability to discriminate between anti-Jo-1 and anti-La antibody specificities: the Jo-1 and La antigens have very similar molecular weights, and there is a risk of misreading results produced by IP or IB. However, the technique is rather less sensitive than IP, and results can be difficult to visualize and therefore interpret.

Method 2.3.1: Ouchterlony double immunodiffusion

An adaptation of the method described by Isenberg & Maddison (1987) (156) was used. A solution of 1% (w/v) agarose (type I) in PBS was slowly heated to boiling point. After cooling to ~35°C, 5 µg/ml penicillin/streptomycin solution (10 000 IU/ml and 10 000 µg/ml respectively) was stirred in, and gels were poured immediately onto glass plates (80 mm x 80 mm), previously arranged on a levelling table. Each plate was evenly coated with 12.0 ml of molten agarose which was allowed to solidify at room

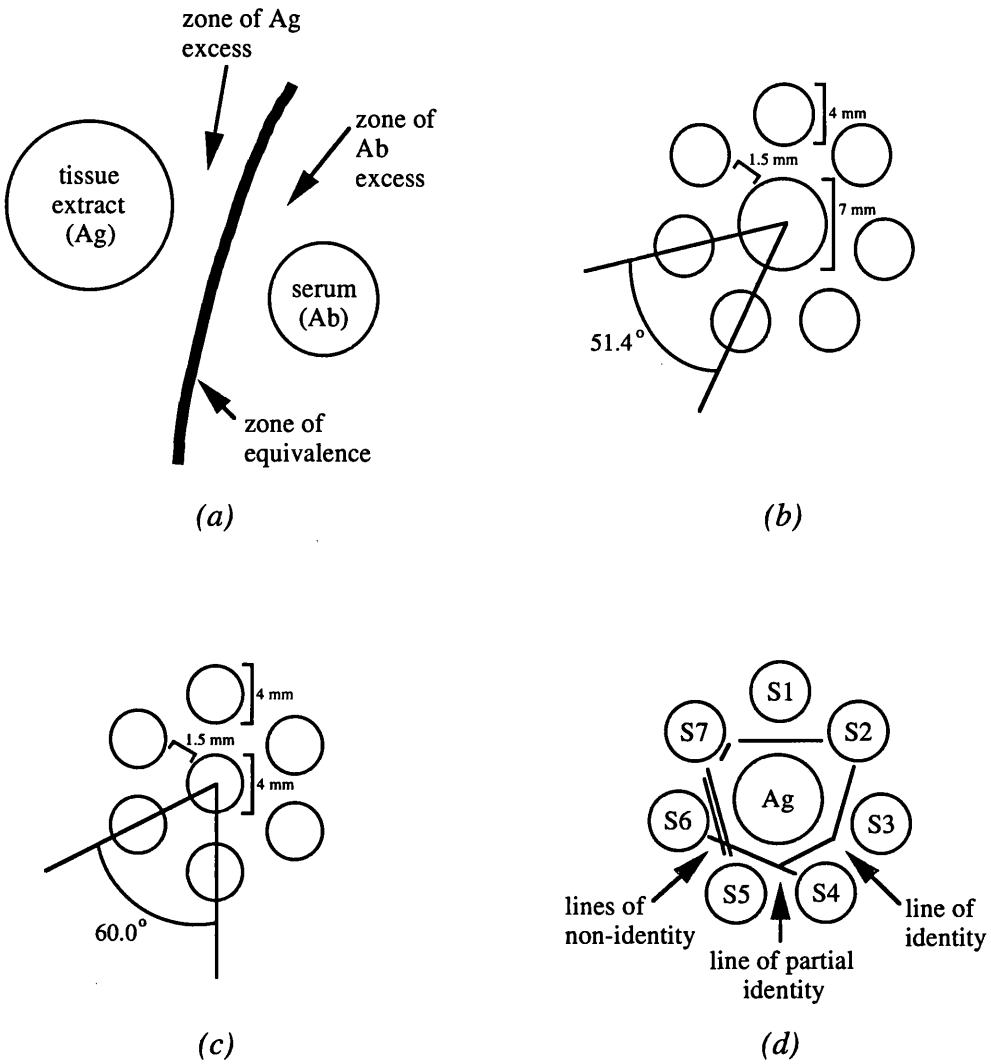


FIGURE 2.1 Detection and identification of autoantibodies by Ouchterlony double immunodiffusion. The technique is based on the formation of an insoluble immune complex in a horizontal agarose gel when an antigen (Ag) diffusing from one well is recognized by antibodies (Ab) diffusing from an apposing well (*a*). Wells are arranged in rosettes, consisting of a central tissue-extract well surrounded by six or seven serum wells (*b* and *c*). Sample and prototype sera (S1-S7) are applied as shown (*d*), and the central well is filled with tissue extract as a source of Ag. This results in a visible precipitin line where the serum contains Abs which recognize a component of the extract (S1), or a clear area if no such Abs are present (S2). A line of identity formed by precipitin lines of adjacent wells indicates that both sera contain Abs which recognize exactly the same antigenic particle (S3 and S4), while crossed lines of non-identity show that the two sera contain Abs which recognize quite different specificities (S5 and S6). A line of partial identity (between S4 and S5) means that a particular Ag is recognized by Abs present in both sera, while a separate Ag is recognized by Abs occurring in only one serum (S5). Sera with more than one specificity often show multiple precipitin lines (S6), each of which interacts independently with adjacent precipitins. Sera with very low titres of Ab produce precipitin lines as shown for S7: identification of such specificities is sometimes possible by repeating the experiment, this time making the serum well as large as the Ag well, and increasing sample volume.

temperature for 1 h. Gels were then stored in heat-sealed polythene bags at 4°C for up to 3 weeks.

Immediately before use, circular wells were punched into the gel using standard size 1 and 3 cork borers: seven small wells (diameter 4 mm) surrounded one large well (diameter 7 mm) at a distance of 1.5 mm to form a single rosette (Fig. 2.1*b*). Six such rosettes could be accommodated on each plate. When testing sera against topo I extract (TIE), a central 4-mm well was surrounded by only six outer wells (Fig. 2.1*c*). After removal of agarose plugs by suction, each outer well was loaded with 20 µl of an undiluted test or standard serum before loading the central well with 60-80 µl of the relevant tissue extract (20 µl in the case of TIE).

Once sera and extracts had soaked into the gel, loaded plates were placed inside square petri dishes and left at room temperature for up to 5 days, during which they were examined on a daily basis for the presence of precipitin lines. Gels were viewed against a matt black background with overhead illumination from a strong desk lamp. The positions of lines of identity, partial identity and non-identity (Fig. 2.1*d*) were recorded and interpreted before discarding the gel.

Each serum sample was tested against the following complementary extracts: rabbit thymus extract (RTE; Pel Freez Ltd, Arkansas, U.S.A./Bradshaw Biologicals, Market Harborough, U.K.), containing U1 RNP, Sm, La and Pm-Scl antigens; an in-house preparation of calf thymus extract (CTE; prepared by Mrs. J.James according to a previously described method (156)), which contained Jo-1 antigen; Ro/La Extract (RLE; Biodiagnostics Ltd, Worcester, U.K.), rich in Ro and La antigens, and TIE ('Scl-70 Extract'; Biodiagnostics Ltd) containing topo I.

A number of reference sera of known autoantibody specificity were available from amongst our own patients, including sera containing antibodies to U1 RNP, Sm, Ro, La, topo I, Jo-1 and Pm-Scl. Depending on the tissue extracts used, positive control sera of the relevant specificities were included on each plate to confirm the presence of antigens. Identification of each resulting precipitin line was achieved by sequential testing of the serum sample alongside appropriate prototype sera, with subsequent analysis of precipitin-line interactions (Fig. 2.1*d*).

2.3.2 Indirect immunofluorescence

Technical basis

Adherent human TC cells (HEp-2 cells) are suspended in fresh medium. Drops of suspension are deposited onto glass multiwell microscope slides, and incubated overnight. During this time most cells will adhere to the slide, and will continue to be engaged in cell division. After washing, the process of fixing the cells renders them penetrable by antibodies. A diluted sample of test or standard serum is applied to each well, and autoantibodies against cellular components remain bound after rinsing away the serum solution. Next the cells are incubated with a fluorescein isothiocyanate

(FITC)-conjugated anti-human-immunoglobulin antibody, and re-washed. This allows visualization of any previously bound autoantibody: upon viewing the cells under a fluorescence microscope, characteristic patterns of bright fluorescence indicate the presence of autoantibodies in the original serum sample and the intracellular location of the autoantigens recognized. Most normal human sera give a negative result, where the level of fluorescence observed is minimal.

Method 2.3.2: Indirect immunofluorescent localization

For most of the work reported here, 18-well HEp-2 slides for indirect IF were obtained commercially (Biodiagnostics Ltd; Quadratech Ltd, Epsom, U.K.). Alternatively, 10-well slides were prepared in-house (Method 2.2.3), and the HEp-2 cell-line was maintained as described in Section 2.2.2.

As has been previously described (361), for initial screening purposes, sample and standard sera were first diluted 1:40 in PBS. Slides were put inside square petri dishes on two leaves of water-dampened tissue-paper: this minimized evaporation from antibody solutions. Each well was loaded with 20 µl of a diluted test or standard serum, taking care to cover the whole area of the well. The slides were incubated at room temperature for 30-60 min, after which samples were rinsed away by gently pouring PBS over each slide. Washing was continued by soaking slides in a PBS bath for 5 min, followed by a second rinse and another 5-min soak in fresh PBS.

Meanwhile, a 1:1000 dilution of conjugate (anti-human polyvalent immunoglobulins (IgA, IgG, IgM) FITC-conjugate developed in goat, IgG fraction of antisera; Sigma Chemical Co. Ltd) was prepared in PBS. After shaking excess liquid from each slide, a tissue-paper-wrapped orange stick was used to dry all areas lying between the wells. The cells were not allowed to dry out – 12-µl of diluted conjugate were immediately loaded onto each well, followed by incubation and washing as before, plus a final rinse in distilled water.

Following removal of excess water from around the wells, two drops of mounting medium (2.5% (w/v) 1,4-diazobicyclo-[2,2,2]-octane(triethylene-diamine) (DABCO) in 5% (v/v) PBS in glycerol) were placed on each slide, avoiding air-bubbles, and a suitable cover-slip applied. This was pressed gently until all wells were covered with a very thin layer of mounting medium, and the cover-slip was aligned and stable. The slides were air-dried for 5 min, then clear cosmetic nail varnish was used to secure the edges of cover-slips where necessary. After leaving to harden for about 30 min, slides were read under a fluorescence microscope, or stored at -20°C for up to two weeks.

2.3.3 Immunoprecipitation

Technical basis

The method is based on the specific binding of the Fc fragment of human IgG antibodies to Protein-A, derived from the cell wall of certain strains of *S. aureus*.

Protein-A is isolated, purified and immobilized by covalent binding, via a spacer arm, to highly cross-linked, spherical agarose beads (Protein-A Sepharose). These beads are easily sedimented by standard centrifugation protocols, to allow thorough washing procedures to be carried out. Such products are available commercially, with a variety of spacer-arm lengths (CL-4B, CL-6B etc.).

The Sepharose beads are first incubated with a diluted sample of human serum. Any IgG antibodies present in the serum may then bind to the immobilized Protein-A. The remaining components of the serum, including excess unbound antibodies, are washed away, and the beads are re-incubated with a radiolabelled extract of human TC-cells. After further washing to remove unbound components of the extract, the bound antigens are removed, reduced and denatured, by boiling in standard Laemmli sample buffer. Constituent polypeptides are then separated according to molecular weight in sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels, followed by autoradiographic detection of polypeptide bands. Each precipitated antigen produces a characteristic banding pattern according to the molecular weights of its component subunits. Banding patterns produced by a particular serum are interpreted by comparison with the patterns produced by internal standard sera, included on the same gel.

Method 2.3.3a: Immunoprecipitation of radiolabelled proteins

A modified version of the method of Craft *et al.* (1988) (66) was used to immunoprecipitate ^{35}S-labelled proteins from K562-cell extracts (prepared according to Method 2.2.4*a*), using human antibodies linked to Protein-A Sepharose. Wherever possible, samples were kept on ice or refrigerated during preparation, and all solutions were ice-cold.

Protein-A Sepharose CL-4B (Sigma Chemical Co. Ltd) was weighed into a 5-ml glass vial: 2 mg Sepharose beads were required for each serum sample to be tested. According to the manufacturer's instructions, the product was first washed as follows. Normally, 50 mg of Sepharose beads were weighed, and transferred into a 15-ml centrifuge tube using 5 x 3-ml volumes of IPP. To wash, hydrate and swell the beads, the suspension was incubated at 4°C for 30 min with end-over rotation. The beads were then sedimented in a refrigerated (1°C) centrifuge (Beckman GPR/GH-3.7) for 3 min at 4000 r.p.m. (Centrifuge Protocol 4; approximately 1100*g*), and most of the supernatant was removed using a pipette, taking care not to disturb the Sepharose pellet. Following a second brief wash in 15 ml IPP, the beads were sedimented according to Protocol 4, and resuspended in 12.5 ml IPP. A 500-µl quantity of the suspension was aliquotted into each of 24 1.7-ml microcentrifuge tubes, keeping the beads evenly suspended by capping and inverting the 15-ml tube every minute or so. A 10-µl serum sample was then added to each sample tube, samples were gently mixed by inversion, and incubated with end-over rotation for 2 h (or overnight) at 4°C.

Following incubation, unbound components of the sample serum were removed by repeated washing in IPP: the beads were sedimented by centrifugation in a microfuge (MSE Microcentaur: 10-sec pulse-spin at 13000 r.p.m. at room temperature (Centrifuge Protocol 5)), and each supernatant was carefully removed; the beads were then resuspended in 500 µl IPP by brief vortexing. The spin-wash cycle was repeated until each sample had been spun down four times. Quick-thawed radiolabelled cell extract (100 µl per sample, corresponding to ~1 million cells prior to labelling) was then added, together with 400 µl IPP, and the tubes were re-incubated for 1-2 h as before.

After similar IPP washing cycles, each sample of beads was resuspended in 50 µl Laemmli sample buffer (SDS reducing buffer: 62.5 mM Tris.Cl pH6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol) by brief vortexing, and stored at -80°C for subsequent analysis of precipitated proteins.

Samples were prepared for SDS-PAGE according to Method 2.3.5*a*. A 15- to 30-µl volume of each denatured sample was loaded into a separate well of a 15-well maxi-gel (8-12.5% acrylamide), as described in Method 2.3.5*c*, or a 10-well mini-gel (Method 2.3.5*b*), and run as usual (5-20 µl of a solution of broadrange kaleidoscope molecular weight markers (BioRad Laboratories) was loaded into one well of each gel). Once run, gels were enhanced (Method 2.3.9*a*), dried onto blotting paper (Method 2.3.9*c*), and autoradiographed (Method 2.3.8*d*).

Method 2.3.3b: Immunoprecipitation of ribonucleoproteins

A similar method to that described above (Method 2.3.3*a*) was used to immunoprecipitate RNPs from unlabelled K562-cell extracts (prepared according to Method 2.2.4*c*). Standard precautions were taken to prevent RNAse contamination. Other modifications, as described by Forman, Nakamura, Mimori *et al.* (96), were as follows.

For each sample, 4 mg Sepharose beads and 20 µl serum were added to the 500 µl IPP buffer. Following incubation with serum, the beads were washed in IPP, as usual, three times. After a further spin (Centrifuge Protocol 5), the beads were resuspended in 300 µl NET-2 buffer, and 200 µl quick-thawed K562-cell extract was added (corresponding to ~8 million cells); the samples were then reincubated as usual. Following incubation with cell extract, the beads were washed four times in NET-2 buffer, using standard spin-wash cycles.

Finally, RNA extraction was performed: keeping everything on ice, the beads were resuspended in 300 µl NET-2 buffer to which was added 30 µl sodium acetate (3 M, pH5.2 (acetic acid was used to adjust the pH)), 15 µl SDS (20%, w/v), 2 µl oyster glycogen (Type XI, Sigma Chemical Co. Ltd; 10 mg/ml) and 300 µl phenol:chloroform:isoamyl alcohol (50:50:1, v/v/v; PCA). The tubes were vortexed briefly and centrifuged in an MSE Microcentaur for 2 min at 13 000 r.p.m. (Centrifuge Protocol 6). If two distinct layers had not formed in the sample tube, further, shorter spins were carried out. Working in a fume cupboard, the majority of the upper, aqueous layer of

each sample was carefully removed with a gel-loading tip into 900 µl ice-cold ethanol: the buffy surface between the two liquid layers consisted of precipitated proteins, and was not disturbed. The samples were then gently mixed with the ethanol and left overnight at -20°C to allow precipitation of RNAs.

The following day, samples were centrifuged in a refrigerated microfuge (Heraeus Biofuge Fresco set at 1°C for 10 min at 9000 r.p.m. (Centrifuge Protocol 7)), and the supernatants were removed, taking care not to disturb the tiny white pellet of RNA. The pellets were then washed by adding 300 µl ice-cold 70% (v/v) ethanol, and the tubes were recentrifuged using Protocol 7. The supernatants were again removed from the tiny white pellets, and the tubes were then loaded into a vacuum centrifuge (GyroVap and Refrigerated Solvent Trap, Howe Ltd, attached to a strong vacuum pump) for a period of 20 min. Once dry, the pellets were dissolved in 20 µl RNA sample buffer (10 M urea, 90 mM Trizma base, 90 mM boric acid, 2 mM sodium EDTA, 0.025 (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol) by brief vortex, and stored at -80°C for subsequent analysis of precipitated RNAs.

Samples were prepared for urea-PAGE according to Method 2.3.5*a*. A 10- to 20-µl sample was loaded into a separate well of a 15-well maxi-gel (7.5 or 10% acrylamide), as described in Method 2.3.5*e*, or a 10-well mini-gel (Method 2.3.5*d*) and run as usual. Once run, RNA gels were silver-stained (see Method 2.3.8*e*), fixed (2.3.9*b*), and dried between cellulose sheets (Method 2.3.9*c*).

Method 2.3.3c: Antigen depletion of radiolabelled K562-cell extracts

This method was adapted from a protocol described by Hirakata, Okano, Pati *et al.*. (1993) (144). A batch of ^{35}S-methionine-radiolabelled K562-cell extract was prepared as usual (Method 2.2.4*a*). The cell extract was then specifically depleted of one or more autoantigens using human antibodies linked to Protein-A Sepharose CL-4B.

First, six separate aliquots of 30 mg Sepharose beads were prepared for use as previously described (Method 2.3.3*a*). For two cycles of depletion, three sets of beads were incubated for 1-2 h with the relevant prototype serum (30 mg beads, 75 µl serum and 7.5 ml IPP in each of three 15-ml centrifuge tubes); the other three was similarly incubated with normal human serum, to serve as a control.

Following incubation, unbound components of serum were removed by repeated washing in IPP: the beads were sedimented according to Centrifuge Protocol 4, and each supernatant was carefully removed; the beads were then resuspended in 7.5 ml IPP by gentle inversion. The spin-wash cycle was repeated until each set of beads had been spun down four times. One set of beads incubated with prototype serum ('prototype-coated beads'), and one set of beads incubated with normal human serum ('normal serum-coated beads') were put aside on ice. The remaining four sets of beads were incubated with 3.0 ml of K562-cell extract plus 4.5 ml IPP for 1 h, after which these beads were immediately spun down according to Centrifuge Protocol 4, and the supernatants put on ice. One supernatant (semi-depleted extract) was then re-incubated

for 1 h with the third tube of fresh autoantibody-coated beads. After incubation and centrifugation, the supernatant (depleted extract) and the remaining sample of semi-depleted extract were each incubated with 5 mg fresh preswollen beads in order to remove any excess antibodies. All three extracts (depleted, semi-depleted and original whole-cell extract) prepared from the same batch were then tested for the presence of the autoantigen by the usual IP method. For comparison, similar "depleted" and "semi-depleted" extracts were prepared using a normal human serum. In some cases four cycles of depletion were carried out in an analogous fashion.

Method 2.3.3d: Affinity purification of autoantigens by immunoprecipitation

This method was basically a scaled-up version of Method 2.3.3*a*. A 20-mg quantity of Protein-A Sepharose was pre-swollen in 5 ml IPP buffer in a 15-ml tube. Following incubation at 4°C (50 µl serum in 5 ml IPP), the beads were repeatedly washed and spun down according to Centrifuge Protocol 4. Re-incubation of the beads for 1 h with 1 ml of the unlabelled or radiolabelled K562-cell extract and 4 ml IPP was followed by similar washing cycles. Washed beads were resuspended in 500 µl Laemmli sample buffer, or stored at -80°C for later use.

Samples were prepared for SDS-PAGE according to Method 2.3.5*a*. The 500-µl denatured sample was loaded into a 45-mm-wide well of a 10-12.5% maxi-gel, as described in Method 2.3.5*c*, or a 10-well mini-gel (Method 2.3.5*b*), and run as usual (a solution of 20-30 µl kaleidoscope molecular weight markers (BioRad Laboratories) was loaded into a standard-sized well of the same gel). Once run, gels were processed for transfer to nitrocellulose membranes (Method 2.3.6*a*) for subsequent blotting studies (Method 2.3.7) and, where appropriate, autoradiography (Method 2.3.8*d*).

2.3.4 Antigen purification by immunoaffinity column chromatography

Technical basis

Affinity chromatography refers to chromatographic separation procedures relying on specific interactions of biomolecules (for review see (367)). Where a protein is purified by a specific antibody, the technique is known as immunoaffinity chromatography. This is a three-stage process involving (i) covalent binding of ligand (i.e. antibody) to particles of an insoluble support matrix, which is then packed into a column; (ii) application of an antigen source to the column followed by extensive washing: the desired protein will remain inside the column, having been recognized by the matrix-bound antibody; (iii) the use of extreme elution conditions, which disrupt the bond between antibody and antigen, while leaving the intact matrix-antibody particles behind.

Due to the myriad proteins present in whole human serum, the solid support matrix must be capable of specifically binding antibodies. A suitable matrix for this purpose is Protein A-coated Sepharose CL-4B beads. The serum is first incubated with a slurry of

the beads to allow specific, non-covalent binding of serum antibodies to Protein-A. After the remaining serum components have been washed away, the antibody-Protein A binding is stabilized by direct covalent coupling. This is achieved with a bifunctional coupling reagent such as dimethylpimelidate (DMP). There are two binding groups on the DMP molecule, each of which is capable of binding to free amino groups of proteins. In the majority of cases, a free amino group will be available on both the Protein A molecule and the Fc portion of the Ig molecule to which it is bound. However, any free amino groups on either of the two proteins will react with DMP, and if such groups are in or near the antigen binding site, antigenicity will be lost. Other binding techniques are available however, should the need arise.

Under optimal conditions immunoaffinity chromatography is an extremely effective technique: a 10000-fold purification of starting material is not uncommon, and at least 1000-fold purification is normally achieved. Elution conditions are, however, critical to the success of the procedure, and must be empirically determined for each individual protein-antibody system in advance of large-scale purifications. Antibody affinity and antigen concentration are also key to efficient separation.

Problems can arise when using polyclonal antibody sources, since a single antigen may be bound simultaneously by several different antibodies resulting in very high avidity and consequent difficulties in effective elution. Moreover, each different antigen-antibody bond has its own elution conditions. Multiple binding of single protein molecules can be minimized by ensuring saturation of antibody with antigen.

Method 2.3.4a: Purification of autoantigens by immunoaffinity column chromatography

The coupling of antibodies to Protein A Sepharose CL-4B beads was carried out using an adaptation of a previously described method (367).

Non-covalent binding of serum antibodies to Protein-A Sepharose

A suspension containing 1.3 g Sepharose beads in ice-cold IPP was prepared, as previously described (Method 2.3.3*a*); total volume 50 ml). The beads were resuspended by gentle vortexing, and 650 µl of the relevant serum sample was added. Following a 2-h (or overnight) incubation at 4°C with end-over rotation, the Sepharose beads were spun and washed four times in 40 ml IPP, being centrifuged according to Protocol 4.

Covalent binding of serum antibodies to Protein-A Sepharose matrix

For covalent coupling of bound antibodies to Protein-A Sepharose, the beads were spun and washed twice in 40 ml sodium borate solution (saturated, pH9.0), being centrifuged according to Protocol 4, but at room temperature. After resuspending in 30 ml sodium borate solution, a 260-mg quantity of solid DMP was added to the borate-Sepharose suspension (final concentration 20 mM), and the mixture was incubated for 90 min at room temperature with end-over rotation. The pH was checked before and after

coupling (usually found to be ~pH9.0): a minimum pH of 8.3 is required for the DMP coupling reaction.

The reaction was stopped by washing the beads once (as above) in 40 ml ethanolamine (0.2 mM, pH8.0; room temperature). The beads were then resuspended in 10 ml ethanolamine solution in a 15-ml tube, and the mixture was incubated at room temperature for 2 h with end-over rotation. Finally the beads were centrifuged according to Protocol 4 (at room temperature), resuspended in 10 ml PBS containing 0.01% merthiolate as preservative, and stored at 4°C until use.

Packing the immunoaffinity column

Empty, reusable 10-ml columns (Model C10) were obtained from Pharmacia Biotech AB, Sweden. The columns were fitted with a volume-adjusting device (AC-10 Adaptor, Pharmacia Biotech AB) to give an available bed volume of 1-10 ml (Fig. 2.2). The manufacturer's instructions were followed when filling the column with the antibody-coupled Sepharose beads. A length of the flexible 1-mm-bore tubing supplied was attached to the bottom end of the column via a special sealing ring and screw piece ('column outlet tube', Fig. 2.2). A separate piece of tubing ('column inlet tube') was passed through the adaptor plunger, and attached inside the end of the plunger using a special sealing device: this allowed delivery of solutions to the main column via the column inlet tube (Fig. 2.2).

The Sepharose beads were centrifuged according to Protocol 4, and resuspended in 10 ml PBS. The column outlet tube was opened, by removing the stopper, and the suspension was loaded into the column using a pipette. As the column drained, PBS washings from the 15-ml centrifuge tube were added. When all the beads had been transferred, the column outlet tube was closed. Keeping the column in a vertical position, the beads were allowed to settle. The column was then filled right to the top with PBS, taking care not to disturb the Sepharose bed. The column inlet tube (attached to the adaptor; Fig. 2.2) was opened before slowly pushing the adapter plunger into the column, avoiding the introduction of air bubbles. Displaced liquid from the column was automatically expelled via the column inlet tube. The plunger was pushed down the column until it was about 5 mm above the Sepharose bed, and secured with its screw-pin attachment. The top-piece of the column was attached, and the column inlet tube was closed: the column was then ready for operation. All subsequent steps were carried out in a cold cabinet set at 4°C, and using ice-cold solutions.

Pre-washing the immunoaffinity column

The actual process of immunoaffinity column chromatography was carried out by an adaptation of the method of Plunkett & Springer (1986) ((269): see also Springer (1993)(324)). The free end of the column inlet tube was attached (using the special sealing ring and screw-piece) to a 30-ml reservoir positioned several cm above the affinity column (Fig. 2.2) The following solutions were then used to wash the column, and to prepare the beads for exposure to the cell extract:

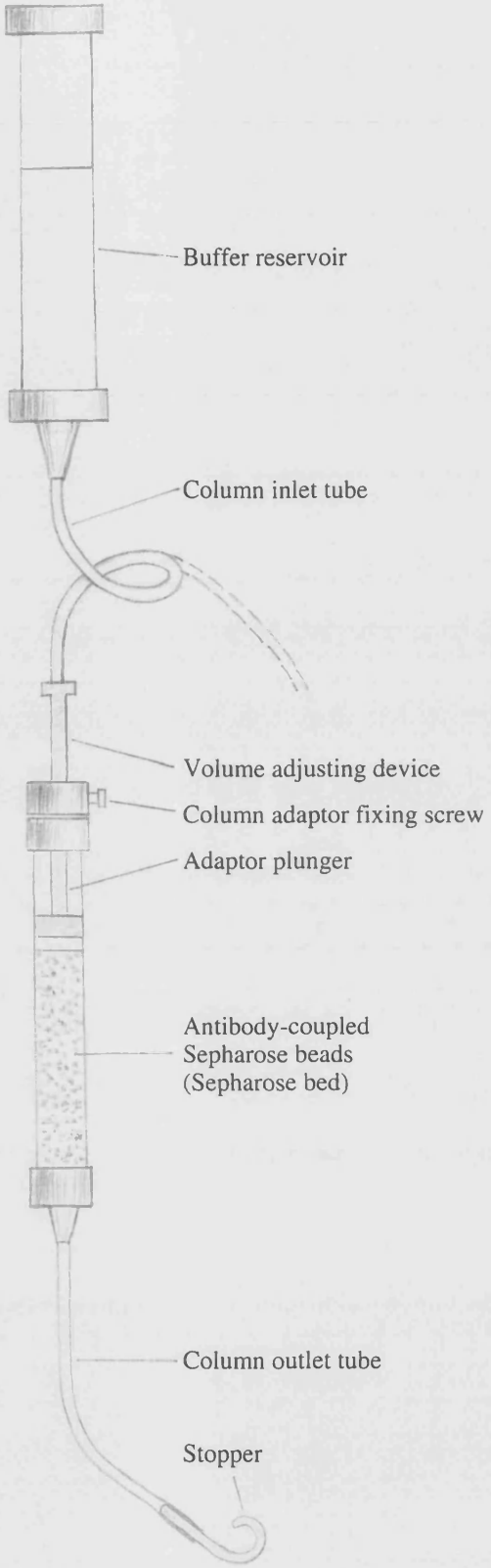


FIGURE 2.2. Schematic representation of immmunoaffinity column chromatography

(i) 50 ml Wash Buffer
(10 mM Tris.Cl, pH8.0; 14 mM NaCl; 0.5% (v/v) Triton X-100)
(ii) 25 ml Tris Buffer, pH8.0
(50 mM Tris.Cl, pH8.0; 500 mM NaCl; 0.1% (v/v) Triton X-100)
(iii) 25 ml Tris Buffer, pH9.0
(50 mM Tris.Cl, pH9.0; 500 mM NaCl; 0.1% (v/v) Triton X-100)
(iv) 25 ml Triethanolamine Solution
(50 mM triethanolamine, ~pH11.5; 150 mM NaCl; 0.1% (v/v) Triton X-100)
(v) 25 ml Wash Buffer

When passing each solution through the column, the following procedure was followed: a 5-ml excess of the relevant solution was poured into the empty reservoir, and the column outlet tube was opened whilst holding it level with the bottom of the column. The end of the outlet tube was positioned above an effluent collection vessel, and its height was adjusted to obtain a satisfactory flow-rate: for washing purposes, this was about 1 drop per second. When the level of the reservoir had dropped to about 5 ml, the column outlet tube was closed, and the reservoir was emptied by inversion, ready for application of the next solution.

Application of cell extract to immunoaffinity columns

In a similar manner to that described above, the cell extract preparation was passed through the two pre-washed columns in series. First, the extract was applied to the preclearing column: the end of the column outlet tube was held above the reservoir before being opened, placed in a clean collection tube, and slowly lowered, to allow a flow rate of approximately 5 ml/h. A series of 15-ml tubes was used to collect 5-ml quantities of the precleared extract. Once the reservoir had virtually emptied, the column outlet tube was closed, and the reservoir was rinsed with Wash Buffer to clean. A 25-ml volume of MIP was then put through the column, in order to flush through the remainder of the cell extract for collection (this was continued until the column contents appeared white, after which the column was closed).

Secondly, the precleared extract was applied to the immunoaffinity column: flow-rate was adjusted to 3 ml/h, as recommended for optimal binding of antigen (324). The 5-ml quantities were applied to the column in the same order as they had been collected, being careful not to allow the reservoir to be completely empty at any time.

Washing the immunoaffinity column

The following solutions were used to wash unbound components of the extract from the column, prior to elution of antigen:

(i) 25 ml Wash Buffer
(ii) 25 ml Tris Buffer, pH8.0
(iii) 25 ml Sodium Phosphate Buffer
(50 mM sodium phosphate, pH6.3; 150 mM NaCl; 0.1% (v/v) Triton X-100)

Low pH elution of bound antigen

The first attempt at eluting bound antigen from the immunoaffinity column was made using a low-pH glycine buffer (50 mM glycine.HCl, pH 2.5; 150 mM NaCl; 0.1% Triton X-100), as recommended (324). A 25-ml quantity of this elution buffer was passed through the column as usual, with a flow rate of approximately two drops per second. A 1.2-ml volume of eluate was collected by hand into each of twenty tubes containing 300 µl of 1 M Tris.Cl, pH9.0.

After use, the immunoaffinity column was washed with 25 ml Wash Buffer, and then filled with PBS containing 0.01% merthiolate for long-term storage at 4°C. A 1-µl quantity of preservative solution (2% sodium azide) was added to each fraction of eluate, and tubes were stored at 4°C until analysis.

Analysis of fractions for eluted antigen

A quick assessment of the relative protein content of each eluted fraction was made using SDS-PAGE followed by silver-salt staining: a 20-µl sample of each fraction was added to 20 µl SDS-reducing buffer and prepared as usual (Method 2.3.5*a*). Twenty-µl samples were run on 10%-acrylamide SDS-PAGE mini-gels (Method 2.3.5*b*), which were then silver stained (Method 2.3.8*c*), fixed (Method 2.3.9*b*) and dried between cellulose membranes (Method 2.3.9*c*). Each well was visually assessed for the presence of a prominent band of the appropriate molecular weight: the fractions judged to have the highest yield were selected for further purification (usually four or five fractions).

Method 2.3.4b: Concentration and desalting of antigen-rich immunoaffinity fractions

Microfuge filtration units (Ultrafree-MC NMWL:30K PLTK: 400-µl volume, 0.2-cm^2 low protein-binding membrane; Sigma Chemical Co. Ltd) were used to de-salt and concentrate antigen-rich immunoaffinity fractions. Each concentrator had a nominal exclusion value of 30 kDa. A 400-µl fraction of eluate was loaded into the concentrator, and the tube was spun in a refrigerated (1°C) microcentrifuge (Biofuge Fresco; Hereaus Instruments) at 9000 r.p.m. for 10 min (Centrifuge Protocol 7; approximately 4000***g***, as recommended by the manufacturers). Following centrifugation, the antigens of interest, having a molecular weight above 30 kDa, were contained in the remaining supernatant of the upper chamber, while salts and water were contained in the lower chamber. The upper chamber was topped up with eluate, while the lower chamber was emptied, its contents being discarded. This process was repeated until all the eluate had been loaded into the filtration unit, and similar 5- or 10-min centrifugation cycles were repeated until the upper chamber contained 25-50 µl of supernatant. This was removed into a 0.5-ml microfuge tube, to which an equal volume of Laemmli sample buffer was added: the sample was immediately prepared (Method 2.3.5*a*) for running on a 10%-acrylamide SDS-PAGE mini-gel in a 30-mm-wide well (Method 2.3.5*b*), after which it was transferred to a PVDF membrane (Method 2.3.6*b*). The PVDF membrane was then stained with Coomassie blue (Method 2.3.8*b*), the band of interest was excised with a

scalpel blade, and sent to a commercial sequence analysis service (Alta Bioscience, University of Birmingham, U.K.) for protein sequencing.

Method 2.3.4c: Protein sequencing

Protein sequencing was carried out at Alta Bioscience by the classic Edman degradation method. This involves labelling of the NH_2-terminal residue of the protein with phenyl isothiocyanate, thus forming a phenylthiocarbamoyl derivative. This is followed by mildly acidic cleavage of the modified NH_2-terminal residue to form a cyclic phenylthiohydantoin amino acid, plus the original polypeptide chain shortened by one residue. This whole procedure is repeated for a number of cycles, and the phenylthiohydantoin amino acids produced are detected sequentially using an in-line high-performance liquid chromatographic analysis system.

Once the NH_2-terminal sequence is known, a variety of protein sequence databases is available, and these can be accessed via servers on the World-wide Web (e.g. the Blitz server: Blitz@ebi.ac.uk). By providing details of sequenced residues, and the position of the sequence (if known, e.g. NH_2-terminal), programmes are available to search these databases for any protein which matches the stipulated sequencing criteria. If enough residues have been sequenced, the identity of the unknown protein may be matched with a small number of candidate proteins, ideally a single protein.

While many proteins can be directly sequenced following purification, over 50% of naturally occurring proteins are found to be blocked at the NH_2-terminal due to post-translational modifications such as acetylation, phosphorylation, formylation, cyclization or poly(ADP-ribos)ylation (62), and such proteins are incompatible with standard sequencing protocols. This situation is immediately apparent to the operator during a sequencing run, due to the production of a characteristic output signal.

Method 2.3.4d: Cyanogen bromide-digestion of NH_2-terminally blocked proteins

Where proteins were reported to be NH_2-terminally blocked, it was necessary to repeat the immunoaffinity purification procedure, break down the resulting protein into a number of polypeptide fragments, and attempt to sequence these instead. A number of protocols have been described to achieve this aim, one of which is "digestion" with cyanogen bromide (CNBr), which cleaves proteins at the COOH-terminal of each methionine residue, and forming a modified COOH-terminal of homoserine lactone in its place: most proteins contain a small number of methionine residues.

In such cases, following identification of antigen-rich fractions eluted from the immunoaffinity column, a more thorough desalting/concentration stage was carried out. To this end, a different type of filtration unit was used (Centriplus-100), obtained from Amicon Ltd (Stonehouse, Gloucestershire, U.K.). All proteins purified by the immuno-affinity method in the present study had a molecular weight of at least 110 kDa. The filtration units had a 15-ml capacity, and a nominal exclusion value of 100 kDa via a 2.34-cm^2 YM hydrophilic cellulose membrane (i.e. all contaminating proteins of below

100-kDa would be removed with these filters, and the inadvertent detection and sequencing of such contaminants would thus be avoided).

The pooled antigen-rich eluate fractions (~7.2 ml in total) were loaded into the concentrator, and the tube was spun in a refrigerated (1°C) centrifuge (Jouan, BR4i; S40 rotor) at 4100 r.p.m. for repeated periods of 5-20 min (Centrifuge Protocol 8; ~3000*g*, as recommended by the manufacturers). Following centrifugation, the antigen of interest was contained in the remaining supernatant of the upper chamber, while salts and water were contained in the lower chamber. Centrifugation was continued until about 30 µl of supernatant was remaining: this process took about 2 h, and frequent progress checks were made to avoid the total elimination of the aqueous phase. The remaining solution was recovered, as advised by the manufacturers, by a 4-min inverted spin at 3400 r.p.m. (~2000*g*) in the same centrifuge (1°C; Centrifuge Protocol 9), the sample collected in the receptacle provided, and transferred to a 0.5-ml microfuge tube.

The previously described CNBr cleavage method of Cook was used (62), with modifications as advised by Dr J.Fox of Alta Bioscience (pers.comm.). All procedures were carried out in a high-performance fume cupboard, and nitrile gloves, and goggles were worn (CNBr is extremely toxic and volatile). A 70-µl quantity of formic acid was added to the sample tube, followed by two small crystals of CNBr. The tube was vortexed, and left in the dark in the fume cupboard for 16-20 h (room temperature).

The next day the tube was again vortexed. The same precautions were taken, and all equipment was enclosed in the fume cupboard. The more volatile components (i.e. water, CNBr, formic acid) were evaporated using a vacuum centrifuge (GyroVap and Refrigerated Solvent Trap, Howe Ltd, attached to a strong vacuum pump) until about 10-20 µl of solution remained. A 200-µl volume of water was then added, and further evaporation was carried out as before. This was repeated using two further 200-µl quantities of water, after which the sample was dried to completion, and 30 µl SDS-PAGE sample buffer was added to the dried residue, theoretically containing the CBr-fragmented polypeptide products of the purified protein.

The sample was immediately prepared (Method 2.3.5*a*) for running on a 10%-SDS-PAGE mini-gel in a 15-mm-wide well (Method 2.3.5*b*), after which it was transferred to a PVDF membrane (Method 2.3.6*b*). The PVDF membrane was then stained with Coomassie blue (Method 2.3.8*b*): any clearly visible bands were to be excised with a scalpel blade, and sent to Alta Bioscience for protein sequencing.

All equipment was thoroughly decontaminated using hypochlorite-type bleach followed by a good rinsing in water (this process oxidizes cyanides to much less toxic cyanates).

2.3.5 Polyacrylamide gel electrophoresis

Technical basis

Proteins are denatured using Laemmli sample buffer, resulting in loss of primary, secondary, tertiary and quaternary structure. The SDS component of the sample buffer coats the unravelled polypeptides with a negative charge. After loading into a polyacrylamide gel (which acts as a molecular sieve), the polypeptides are attracted by the application of a positive charge potential, applied via a Tris-glycine buffer system, and the extent of their migration is negatively correlated with their molecular weights. This results in bands of protein, which may be detected by a variety of methods.

A similar principle is used to separate RNAs on the basis of their length using a urea-acrylamide gel, and a TBE buffer system.

Method 2.3.5a: Sample preparation for polyacrylamide gel electrophoresis

The sample, prepared by IP or immunoaffinity column chromatography, had been placed in a tube capable of withstanding boiling temperatures, which had an air-tight, secure closing mechanism (screw-top poly-propylene tubes and polypropylene microfuge tubes fitted with lid-locks are suitable for this purpose).

After adding an appropriate quantity of sample buffer to each sample (refer to individual protocols), lids were securely fitted, tubes were placed into floating racks, and the racks were floated on boiling tap water for 3 min (water at 65°C in the case of RNA samples), such that the samples were under the surface of the water, while the lids were protected from submersion. The samples were allowed to cool for 5 min at room temperature. Where Protein-A Sepharose beads were present in the sample, tubes were centrifuged according to Protocol 5.

Method 2.3.5b: SDS-PAGE of proteins on a mini-gel

Standard mini-gel electrophoresis equipment (Modular Mini-Protean II Electrophoresis System (Bio-Rad); Mighty Small II/SE250 (Hoeffer Scientific Instruments, San Francisco, CA, U.S.A.)) was used to separate denatured polypeptides according to their molecular weight.

<u>Assembly of gel-casting unit</u>

The equipment was assembled according to the manufacturer's instructions: for each gel, two clean, ethanol-polished glass plates (~80 x 100 mm) were placed one on top of the other, the shorter edges being separated by two 1.0-mm-thick Teflon spacers (~10 x 80 mm) such that a space of ~1.0 x 70 x 80 mm was left in the middle. The plates and spacers were carefully aligned to avoid leaks, and the sandwich was gripped in place by casting clamps. The lower open end of this assembly was locked tightly onto the casting stand on top of a casting gasket, such that a water-tight space was formed between the two plates for casting the gel.

Gel composition and pouring

The gel components were assembled as follows: 10% (w/v) ammonium persulphate (AMPS; Bio-Rad Laboratories) solution which was prepared freshly each day; N,N,N',N'-tetramethylethylenediamine (TEMED) was also obtained from Bio-Rad Laboratories; solutions of 1.5 M Tris.Cl, pH8.8; 0.4% (w/v) SDS (Lower Gel Buffer) and 0.5 M Tris.Cl, pH6.8; 0.4% (w/v) SDS (Upper Gel Buffer) were prepared well in advance, and stored at 4°C; 30% (w/v) acrylamide : bisacrylamide (37.5:1) was obtained from nbl Gene Sciences Ltd, Cramlington, U.K, also being stored at 4°C.

The recipes used to prepare two SDS-PAGE mini-gels of the appropriate percentage are shown in Table 2.1*a*. First, the lower gel components were added to a 50-ml polypropylene tube in the order shown, with the AMPS and TEMED being added just before the gel was ready to be poured. The tube was closed and gently inverted three times to mix, after which ~3-4 ml of the mixture was poured carefully between the glass plates to a height of ~50 mm, avoiding air bubbles. Immediately after being poured, a 100-µl volume of water-saturated butan-2-ol (30 ml butan-2-ol and 20 ml water were shaken and allowed to equilibrate and separate for 24 h: the top layer was then ready for use) was carefully introduced via a gel-loading tip, which was whisked along the gap between the plates, taking care not to touch the gel directly.

The gel was left to polymerize for at least 45 min, after which the butan-2-ol and any surplus water was washed away with several pourings of water and shaking. The apparatus was then shaken well to clear its surface, ready to pour the upper gel: this was prepared in much the same way as the lower gel (see Table 2.1*a*). The upper gel was poured between the glass plates on top of the lower gel, avoiding air bubbles, until the gap between the plates was full. Immediately, an appropriate Teflon comb was inserted between the glass plates, displacing some of the upper gel mixture, and such that the teeth of the comb were ~5 mm above the surface of the lower gel. It was checked that the comb was parallel with the top of the lower gel, and any small air bubbles could usually be removed by gentle tapping of the glass plates.

The gel was left to set for at least 30 min, after which the comb was carefully removed in a vertical direction, and the wells were immediately washed out with water, as before.

Running conditions

The gel clamps were immediately removed from the casting stand, and clipped into the electrophoresis apparatus assembly. The upper and lower buffer chambers thus formed (between two separate gels) were then filled with freshly made running buffer (25 mM Trizma base, 190 mM glycine, 0.1% (w/v) SDS), prepared by diluting a 5x concentrate (125 mM Trizma base, 950 mM glycine, 0.5% (w/v) SDS) stored at 5°C .

Appropriate sample volumes (see individual protocols) were then carefully loaded into separate wells using a gel-loading pipette tip: great care was taken not to overfill

TABLE 2.1. Recipes used for preparing polyacrylamide gels for polyacrylamide gel electrophoresis (PAGE): (*a*) sodium dodecyl sulphate- (SDS-) PAGE mini-gel recipes; (*b*) SDS-PAGE maxi-gel recipes; (*c*) urea-PAGE mini-gel recipes; (*d*) urea-PAGE maxi-gel recipes

(*a*)

	Proportion of acrylamide in gel mixture (%)				
	5.0*	8.0	10.0	12.5	15.0
Component volume (ml)					
Milli-Q water	3.9	4.8	4.2	3.3	2.5
Lower gel buffer**	-	2.5	2.5	2.5	2.5
Upper gel buffer***	1.7	-	-	-	-
Acrylamide mixture†	1.1	2.7	3.3	4.2	5.0
AMPS§	0.04	0.07	0.05	0.04	0.03
TEMED¶	0.01	0.02	0.01	0.01	0.01
Total volume (ml)	~6.7	~10.0	~10.0	~10.0	~10.0

(*b*)

	Proportion of acrylamide in gel mixture (%)				
	5.0*	8.0	10.0	12.5	15.0
Component volume (ml)					
Water	11.8	14.5	12.5	10.0	7.5
Lower gel buffer*	-	7.5	7.5	7.5	7.5
Upper gel buffer**	5.0	-	-	-	-
Acrylamide mixture†	3.2	8.0	10.0	12.5	15.0
AMPS§	0.10	0.21	0.15	0.11	0.09
TEMED¶	0.03	0.06	0.03	0.02	0.02
Total volume (ml)	~20.0	~30.0	~30.0	~30.0	~30.0

(*c*)

	Proportion of acrylamide in gel mixture (%)	
	7.5	10.0
Component volume (ml)		
Milli-Q water	3.4	2.6
TBEx5*	2.2	2.2
Urea (mass in g)	4.6	4.6
Acrylamide mixture†	2.8	3.6
AMPS§	0.08	0.06
TEMED¶	0.02	0.02
Total volume (ml)	~13.0	~13.0

(*d*)

	Proportion of acrylamide in gel mixture (%)	
	7.5	10.0
Component volume (ml)		
Water	6.9	5.2
TBEx5*	4.4	4.4
Urea (mass in g)	9.2	9.2
Acrylamide mixture†	5.5	7.3
AMPS§	0.15	0.12
TEMED¶	0.04	0.04
Total volume (ml)	~26.0	~26.0

*Upper gels were always 5% for SDS-PAGE
**1.5M Tris.Cl pH8.8; 0.4% (w/v) SDS
***0.5M Tris.Cl pH6.8; 0.4% (w/v) SDS
†30% (w/v) acrylamide : bisacrylamide (37.5:1)
§ 10% ammonium persulphate (w/v)
¶ N,N,N',N'-tetramethylethylenediamine
#Tris-borate-EDTA x5 (450 mM Trizma base, 450 mM boric acid, 10 mM disodium EDTA)

wells, and cross-contamination of wells was avoided. Molecular weight markers (5-30 µl, as appropriate) were similarly added to a separate well (see individual protocols).

The lid assembly was put into place, and electrodes were attached to a suitable power pack: two mini-gels were typically run at a constant voltage of 75 mV. When the blue dye front was observed to be fully below the upper gel, the voltage was increased to 125 mV. When the dye front had virtually reached the bottom of the lower gel (~60-90 min) the power was switched off, the whole unit was carefully dissembled, and the glass plates were prised apart, the gel then being ready for further processing, as described in individual protocols.

Method 2.3.5c SDS-PAGE of proteins on a maxi-gel

Standard maxi-gel electrophoresis equipment (Protean II xi Vertical Electrophoresis Cell (Bio-Rad Laboratories); SE600 Gel Electrophoresis System (Hoeffer Scientific Instruments)) was used to separate denatured polypeptides according to molecular weight.

Assembly of the gel-casting unit

The plate sizes were ~200 x 200 mm (Bio-Rad Laboratories) and ~160 x 140 mm (Hoeffer Scientific Instruments), and spacers were also larger.

Gel composition and pouring of gels

The process was essentially the same as described for mini-gels, with the following modifications. The recipes used to make the maxi-gels are shown in Table 2.1*b*. A larger (200 µl) volume of butan-2-ol was overlaid on the lower gel.

Running conditions

The same basic method was used, except the two maxi-gels were run at constant current – 48 mA to start with, being increased to 72 mA when the dye front had completely passed through to the lower gel. In addition, both types of equipment included cooling coils through which cold running tap-water was allowed to flow. Total running time was ~3-5 h depending on how far the dye front was allowed to travel down the lower gel: this was determined largely according to convenience.

Method 2.3.5d: Urea-polyacrylamide gel electrophoresis of RNAs on a mini-gel

The electrophoretic separation of RNAs was carried out using the same equipment as for protein electrophoresis (Method 2.3.5*b*), and a similar basic technique was used. Standard precautions were taken to avoid contamination by RNAse. All modifications are detailed below.

Gel composition and pouring of gels

The gel components were as follows: 10% (w/v) AMPS (freshly prepared each day); TEMED; a 5x solution of Tris-borate-EDTA (TBE: 450 mM Trizma base, 450 mM boric acid, 10 mM disodium EDTA) was prepared well in advance, and stored at 4°C; 30% (w/v) acrylamide : bisacrylamide (30:1) was obtained from Sigma Chemical Co. Ltd, also being stored at 4°C.

The recipes used to prepare two 7M-urea-polyacrylamide mini-gels of the appropriate acrylamide percentage are shown in Table 2.1*c*. The gel components were added to a 50-ml polypropylene tube in the order shown, with the AMPS and TEMED being added just before the gel was ready to be poured. The tube was closed and gently inverted three times to mix, after which the gel was poured between the glass plates, avoiding air bubbles, until the gap between the plates was full. Immediately, an appropriate Teflon comb was inserted between the glass plates, displacing some of the upper gel mixture, and such that the teeth of the comb were fully immersed in the unpolymerized gel solution. It was checked that the comb was parallel with the top of the lower gel, and any small air bubbles could usually be removed by gentle tapping of the glass plates.

Each gel was left to set for at least 60 min, after which the comb was carefully removed in a vertical direction, and the wells were immediately washed out with water, as usual.

Running conditions

The upper and lower buffer chambers were filled with freshly made running buffer (TBEx1).

Markers were not used: relative mobilities were compared by the use of internal standards i.e. RNA samples prepared using prototype sera.

Two mini-gels were typically run at a constant current of 12 mA for 1 h: this meant the gels were heated to a temperature of ~50-60°C during the run. When the first dye-front had virtually reached the bottom of the lower gels (~60-90 min) the power was switched off, the whole unit was carefully dissembled, and the glass plates were prised apart, the gels then being ready for further processing, as described in individual protocols.

Method 2.3.5e: Urea-polyacrylamide gel electrophoresis of RNAs on a maxi-gel

Gel composition and pouring of gels

The process was essentially the same as described for mini-gels. The Hoeffer system was used. The recipes used to make the maxi-gels are shown in Table 2.1*d* .

Running conditions

The same basic method was used, except two gels were run at a constant current of 40 mA, until the samples had entered the gel, when the current was raised to 60 mA (in the absence of cooling coils, this permitted a running temperature of ~50-60°C). Total running time was ~1-2 h.

2.3.6 Electrophoretic transfer

Technical basis

Polypeptides are separated according to molecular weight by SDS-PAGE. The SDS-polyacrylamide gel is then laid onto a sheet of nitro-cellulose (or other suitable

membrane). The gel and membrane are sandwiched between sheets of blotting paper and Scotchbrite pads and put inside a special cassette. All components of the gel sandwich are kept wet with a special electrophoretic transfer buffer throughout the process of sandwich assembly.

The cassettes are loaded into a transfer buffer-filled electrophoretic transfer tank in such a way that, during application of an electric current, the negatively charged SDS-coated polypeptides are attracted to the positive anode. The sandwich is constructed so the polypeptides will consequently move across from the gel to the membrane. The composition of the membrane is highly attractive to polypeptides, which will bind and remain adhered to the membrane surface, even after membrane washing. Because the proteins pass straight across, the banding pattern is essentially analogous to that of the original gel. Coloured molecular weight markers on the surface of the membrane from the original gel allow visual confirmation of a successfully transferred gel.

Choice of membrane type depends on application. Nitro-cellulose membranes are cheap, and are useful for routine blotting procedures. However, they are also fragile, difficult to stain with Coomassie blue, and unsuitable for protein sequencing applications. Polyvinylidene fluoride (PVDF) membranes (Immobilon-P^{SQ}; Millipore) are more expensive, but are also more robust, than nitro-cellulose membranes, and are suitable for use in protein sequencing applications and when Coomassie blue staining is required. A further disadvantage of PVDF is that it must be pre-wetted before use in aqueous solutions, and must not be allowed to dry out during a subsequent procedure (to do this the membrane is soaked in methanol for a few seconds, until grey in colour, then washed in water for two minutes to elute the methanol, making sure the membrane is fully submerged at all times).

Method 2.3.6a: Electrophoretic transfer onto nitro-cellulose membranes

Standard equipment was used, obtained from BioRad Laboratories (Mini Trans-Blot Electrophoretic Transfer Cell; Trans-Blot Electrophoretic Transfer Cell). Maxi-gel and mini-gel transfers were essentially the same.

First, transfer buffer (20 mM Trizma base, 150 mM glycine, 20% (v/v) methanol, 0.1% SDS) was freshly prepared using a 10x concentrate of Transfer Buffer Mixing Solution (200 mM Trizma base, 1.5 M glycine). Eight pieces of blotting paper, a little larger than the gel itself, were soaked in transfer buffer, together with four similarly sized pieces of Scotch-Brite pad, and two pieces of pre-wetted nitro-cellulose membrane (BioRad Laboratories), each a little larger than a gel.

Following removal of the gels from the glass plates after SDS-PAGE, each gel was equilibrated in Transfer Buffer for 10 min. The two gel sandwiches were then assembled "underwater", each comprizing the following layers: Scotchbrite pad x 1, blotting paper sheet x 2, SDS-polyacrylamide gel x 1, transfer membrane x 1, blotting paper sheet x 2, Scotchbrite pad x 1. It is extremely important to avoid air bubbles, especially between the gel and the membrane, in order for effective transfer to take

place. Each sandwich was placed in a transfer cassette, and immediately afterwards the two cassettes were fitted into the tank apparatus in an appropriate orientation, according to the manufacturer's instructions. The tank was filled to the brim with transfer buffer, the tank lid was fitted, and the current was switched on: 215 mA constant current was used for both mini- and maxi-gels. For mini-gels, the partly submerged ice-pack cooling method was used (a suitable vessel being provided by the manufacturer), while maxi-gels were cooled by attaching cold-water taps to the cooling coils provided.

After 2 h had elapsed, the apparatus was partly dissembled so a small corner of the membrane could be checked for effective transfer of coloured molecular weight markers: if transfer was incomplete the apparatus could be re-assembled and run again for a while. Following transfer, gels were discarded, and membranes were briefly rinsed in water, stained with Ponceau S to check for protein content (Method 2.3.8*a*) and allowed to dry, ready for use. Membranes could be stored at room temperature for several days, but temperatures of -20°C were used for longer periods of storage, with the membrane being carefully wrapped in aluminium foil.

Method 2.3.6b: Electrophoretic transfer onto PVDF membranes

The same basic method as that described above was used, with the following modifications: (i) the SDS-polyacrylamide gel used was 24-36 h old to prevent amino-acid modification due to non-polymerized acrylamide monomer; (ii) an alternative transfer buffer was used, as recommended (62) (25 mM Tris, 192 mM glycine, 10% (v/v) methanol), (iii) after removal of the PVDF membrane from the transfer cassette, the membrane was repeatedly washed in water over a 10-min period to remove all contaminating traces of Tris and glycine.

2.3.7 Immunoblotting

Technical basis

The technique of IB relies on antibodies which recognize linear epitopes. A disadvantage of IB is that it can only be used to detect antigenic epitopes of primary structure, which are still intact on the denatured particle.

Whole-cell extracts do not contain sufficient quantities of nucleolar antigens for effective detection of antinucleolar antibodies by IB. However, appropriate sera may be used to affinity purify autoantigens from cell extracts by scaled-up IP techniques, thus enabling IB studies to be performed using the purified protein extracts as the antigen source.

The purified source of antigen is run on SDS-PAGE, followed by electrophoretic transfer to a nitro-cellulose membrane. The membrane is cut into strips which are incubated with a diluted sample of serum. Autoantibodies recognizing linear epitopes then bind to the denatured antigens. After washing, the strips are incubated with an alkaline phosphatase-conjugated anti-human immunoglobulin. After re-washing, the

samples are incubated with appropriate alkaline phosphatase substrates, which results in a coloured product in the vicinity of bound conjugate. Bands are thus visualized, which are indicative of the molecular weights of the particular antigenic subunits recognized by autoantibodies in the sample serum.

Method 2.3.7: Immunoblotting of purified autoantigens

Immunoblotting was performed by a modified version of the previously described method of McHugh, James & Maddison (1988) (225) using sample sera and prototype sera of known autoantibody specificities.

Preparation of blotting strips

Membranes, prestained with Ponceau S, were cut into 3- to 5-mm-wide strips with a clean scalpel blade, perpendicular to the dye front: the orientation of the transferred proteins on the membrane was aided by pencil marks made on the membrane immediately following transfer (the transferred gel had been traced around), and the Ponceau S staining helped make sure the strips were cut perpendicular to the linear patterns of electrophoresed proteins. A couple of cm of clean membrane were left at either end of each strip to aid handling (membranes were handled using clean tweezers). A pencil was used to mark a reference number on one end of each strip.

Incubation of blotting strips with sample sera

A standard 20-well blotting tray was used (BioRad Laboratories) for separately incubating strips in 2 ml fat-free milk, freshly reconstituted from a dried domestic skimmed milk powder as a 5% solution in PBS (Blotto): this served to block non-specific protein-binding sites. The blotting tray was put on a moving platform during all incubations. After 2 h, the blotto was removed, and immediately replaced with 2 ml fresh blotto. Typically, a 2-20 µl sample of each sample serum was then added to the relevant well (i.e. 1/1000 - 1/100 dilution), and the samples were incubated at room temperature for a further 60-90 min. A range of prototype sera were always included to act as positive and negative controls during colour development, and to act as internal standards for the purpose of band identification. The strips were then washed in three separate lots of fresh PBS over a 10-min period.

Binding of the second antibody (anti-human IgG/IgM developed in goat, conjugated to alkaline phosphatase; Sigma Chemical Co. Ltd) was added at a typical concentration of 1/1000 - 1/250 (2-8 µl in 2 ml blotto per well). After further washes as before, the strips were ready for developing.

Developing immunoblotting strips

A 100-ml quantity of developing buffer (100 mM Tris.Cl, 100 mM NaCl, 5 mM magnesium chloride; pH9.6) was placed in a clean, white, polypropylene tray. The substrates were then added: 200 µl 5-bromo-4-chloro-3-indolyl phosphate solution (BCIP; 50 mg/ml in dimethylformamide (DMF)) and 200 µl nitroblue tetrazolium solution (NBT; 50 mg/ml in 70% (v/v) DMF), and the substrate preparation was mixed by swirling. The strips were briefly rinsed in developing buffer, and then placed into the

incubation tray. All strips were placed with transferred proteins facing upwards, and any overlapping strips were gently moved with tweezers. The tray was left for 5-20 min at room temperature, with very frequent checks being made for colour development. When optimal contrast between positive bands and negative background was judged to have been achieved, the reaction was halted by removing all strips into a large tray of water. After 2-3 min the strips were laid out onto blotting paper and allowed to air-dry.

Autoradiography

Immunoblots were also subject to autoradiography where appropriate (Method 2.3.8*d*) to reveal the positions of transferred polypeptides.

2.3.8 Protein and RNA detection methods

A variety of methods is available for the detection of proteins in polyacrylamide gels and/or on membranes, and the method of choice depends on the particular application.

Ponceau S staining is the easiest method, and also has the advantage of being reversible. However, the method lacks sensitivity.

Coomassie blue staining is typically used for the detection of μg quantities of protein in SDS-polyacrylamide gels. However, an adapted technique can also be used to detect proteins which have been transferred from acrylamide gels onto PVDF membranes, and this method is considerably more sensitive than Ponceau S staining, allowing detection of 100-200 ng of protein (62).

Staining of proteins using silver-salt staining is a very sensitive technique capable of detecting proteins in acrylamide gels at the ng level (62). However, the method is long-winded, technically more difficult and more hazardous.

Autoradiography is probably the most sensitive technique, but it relies on radiolabelled proteins, and is thus technically difficult and more hazardous. If signals are weak, then films can be exposed for several weeks by this method. However, background staining will also accumulate during such long exposures, and so samples should be of the highest purity for optimal sensitivity: this amounts to thorough washing cycles during IP procedures (Method 2.3.3*a*).

The detection of RNAs by silver staining is a sensitive technique, and is considerably less hazardous than the alternative, which is autoradiography of ^{32}P-labelled RNAs immunoprecipitated from previously radiolabelled TC cells.

Method 2.3.8a: Detection of proteins by Ponceau S staining

The nitro-cellulose membrane was placed in a suitable tray containing sufficient Ponceau S solution (0.2% Ponceau S, 1% (v/v) acetic acid) for full immersion. The tray was placed on a moving platform, and allowed to stain for 5 min at room temperature. The membrane was then destained with several washings of water over a 5-min period.

Method 2.3.8b: Detection of proteins by Coomassie blue staining

The PVDF membrane was pre-wetted as described in Section 2.3.6*b*, and placed in a suitable tray containing sufficient Coomassie blue solution (0.1% Coomassie blue (w/v), 50% methanol (v/v), 2% glacial acetic acid (v/v)) for full immersion. The tray was placed on a moving platform, and allowed to stain for no longer than 5 min at room temperature (longer staining times result in too much background staining). The membrane was then destained using several changes of 50% methanol over a 10- to 30-min period (destaining was halted when protein bands were clearly visible over the background). The membrane was then rinsed in several washings of water over a 5-min period, and allowed to air-dry.

Method 2.3.8c: Detection of proteins by silver staining

A "Plusone" protein silver staining kit was used, supplied by Pharmacia, and the manufacturer's instructions were carefully followed. Briefly, each gel was carefully removed from glass plates following SDS-PAGE (Section 2.3.5), and placed in fixative solution (4 : 1 : 5, ethanol : glacial acetic acid : water). The container of fixative was placed on a moving platform for 30 min, ensuring there was sufficient quantity of fixative to allow total submersion of the gel throughout this period. The fixative solution was then removed from the container by aspiration, a similar volume of sensitizing solution (0.125% (w/v) glutardialdehyde, 0.2% (w/v) sodium thiosulphate, 830 mM sodium acetate, in 30% ethanol) was added, and the gel was reincubated for a further 30 min. After removal of sensitizing solution, the gel was washed three times for 5 min each time, in water. Next, the gel was soaked in silver solution (0.25% (w/v) silver nitrate, 0.015% (w/v) formaldehyde) for 20 min, followed by two 1-min washes in water. Gels were then developed for 2-6 min in developing solution (240 mM sodium carbonate, 0.0075% (v/v) formaldehyde): once bands had appeared the developer was removed and replaced with stopping solution (39 mM EDTA), for a period of 10 min. Finally, the gel was washed three times for 5 min each with water. The gel was then ready for fixing (Method 2.3.9*b*) and drying between cellulose membranes (Method 2.3.9*c*)

Method 2.3.8d: Detection of S^{35}-labelled proteins by autoradiography

Dried gels or blots containing radiolabelled proteins were taped into autoradiography cassettes and exposed to film (blue-sensitive X-ray film; Genetic Research Instrumentation Ltd, Dunmow, U.K.) for 1-4 weeks, depending on strength of signal.

Method 2.3.8e: Detection of RNA by silver staining

A 'Silver Stain Kit' silver-staining kit was used, supplied by BioRad Laboratories, and the manufacturer's instructions were followed carefully.

Once urea-polyacrylamide gels were run, they were removed from the glass plates, placed in a glass tray containing sufficient 'Fixative Solution 1' (40% (v/v) methanol) to

completely immerse the gel, and the tray was placed on a moving platform at room temperature for 30 min. In a similar fashion, gels were then incubated in 'Fixative Solution 2' (10% (v/v) ethanol) for 15 min, in a fresh quantity of 'Fixative Solution 2' for a further 15 min, and in oxidizing solution (1/10 dilution of 'Oxidizer concentrate' (BioRad Laboratories)) for 5 min. Several water washes were then carried out over a 15-min period. The gels were then placed in the 'Silver Reagent' for 20 min, washed in water for 20-30 s, and then placed in 'Developer Solution' ('Developer' solid (BioRad Laboratories) was dissolved in water: 32 g/l) for ~30 s or until a brown precipitate was visible in the solution. Fresh quantities of 'Developer' were added in this way until an optimal balance between background and positive bands was judged to have been reached. The gel was then soaked in 5% acetic acid for 15 min to stop the reaction. Finally, the gel was fixed (Method 2.3.9*b*) and dried between cellophane sheets (Method 2.3.9*c*).

2.3.9 Processing of gels and membranes

Method 2.3.9a: Enhancement of SDS-polyacrylamide gels for autoradiography

Gels were enhanced as described by Craft *et al.* (1988) (66). After removal from glass plates, SDS-polyacrylamide gels containing radiolabelled samples were soaked in 0.5 M sodium salicylate for 15 min. Gels were then fixed (Method 2.3.9*b*) for 30 min, before drying onto blotting paper (Method 2.3.9*c*).

Method 2.3.9b: Fixing polyacrylamide gels

After removal from glass plates each gel was placed in a tray containing sufficient fixative solution (methanol:water:glacial acetic acid, 4.5:4.5:1) to completely submerge it. The tray was placed on a moving platform for at least 30 min (or overnight).

Method 2.3.9c: Drying polyacrylamide gels

All gels were dried using a Rapidry gel dryer (ATTA Electrophoresis) attached to a Vacuum pump (Pump PV 300, Hoeffer Scientific Instruments U.K., Newcastle-under-Lyme, U.K.). Each gel was placed onto a clean glass plate. A larger piece of blotting paper was then placed over the gel, and gently pressed against it. The blotting paper was slowly peeled away, bringing the gel with it. Alternatively, the gel was placed between two sheets of cellophane (Cellophane Membrane Backing, BioRad Laboratories) before being placed onto filter paper. A larger piece of cling-film was placed over the whole gel and its coverings, before being placed onto the drying platform; the gel-dryer covers were closed, the vacuum applied, and gels were dried at 70°C for about 45-75 minutes.

2.4 STATISTICAL ANALYSES

2.4.1 χ^2-test

All comparisons were performed by χ^2-analysis of 2 x 2 contingency tables. This test is suitable for comparing the distribution of a discrete variable in one sample with the distribution of the same discrete variable in another sample where there are only two possible values of the discrete variable. Using Table 2.2 below as a guide, the value of X^2 (the test statistic) is calculated using the following formula:

$$X^2 = \frac{(ad-bc)^2 . N}{t_1 . t_2 . n_1 . n_2}$$

The significance level of $P<0.05$ was used, and X^2 values were assessed according to the tables of Fisher & Yates (92).

It should be noted, however, that a χ^2-test is inappropriate in cases where $N<20$, or if $20<N<40$ and the smallest expected value is less than five (59) (i.e. the value expected if the Null hypothesis (no difference between the populations) was correct). In cases where the values were small, but appropriate for a χ^2-analysis, a correction factor for continuity was used, as divized by Yates (372). Yates' correction was included when $N<100$, and/or any cell contained a number less than ten. The following formula was used for calculating the value of X^2 when Yates' correction was required:

$$X^2 = \frac{\{(|ad - bc|) - N/2\}^2 . N}{t_1 . t_2 . n_1 . n_2}$$

TABLE 2.2 Standard 2 x 2 contingency table for χ^2-analysis of discrete variables

	Discrete variable		
	Y	**Z**	**Total**
Population 1	a	b	$t_1=a+b$
Population 2	c	d	$t_2=c+d$
Total	$n_1=a+c$	$n_2=b+d$	N

CHAPTER 3

RESULTS, PART 1

Characterization of anti-nucleolar antibody reactivity in systemic sclerosis patients and their relatives

3.0 SUMMARY

The prevalence and specificity of ANAs and ANoAs in 58 SSc probands, four first-degree relatives with SSc, 215 first-degree relatives without SSc, and their 24 spouses were studied. All sera were screened for ANAs, ACAs, and ANoAs by IF. Nuclear and nucleolar autoantigens were then characterized by IP of ^{35}S-methionine-labelled K562-cell extracts; ANAs were also identified by Ouchterlony double immunodiffusion.

Antinuclear-IF was present in 51 patients with SSc (82.3%), in 50 SSc-free relatives (23.3%), and in six spouses (25.0%). Antinucleolar-IF was detected in 25 SSc patients (40.3%), in 33 SSc-free relatives (15.3%), and in four spouses (16.7%). Forty-three sera had autoantibodies to defined nuclear antigens, including eleven with anti-RNAP III, nine with anti-RNAP II, eight with anti-topo I, and 13 with ACAs. All but two were from SSc patients: anti-Ro/La antibodies were detected in two first-degree relatives, one with SLE, and one with PA. Twenty-four sera had autoantibodies to defined nucleolar antigens by IP (seven Pm-Scl, ten RNAP I, four U3 RNP and three Th RNP), and all were from patients with SSc (38.7%). No serum was found to contain more than one defined ANoA. Furthermore, ACAs, anti-topo I antibodies, and each of the defined ANoAs appeared to be mutually exclusive. Four sera had autoantibodies to topo I and RNAP II, one of which also precipitated RNAPs I and III. The disease subtype associations of the defined ANoAs were consistent with previous reports.

Sera from 118 randomly chosen family members without CTDs were then separately compared with 120 age- and sex-matched blood-donor controls to determine whether they had an increased frequency of ANAs or ANoAs to that expected. Antinucleolar-IF was significantly more common in the unaffected first-degree relatives of SSc patients (18.1%), but not in their spouses (7.7%), when compared with controls (8.3%; $P<0.05$). Most of the IF-ANoA-positive sera from relatives displayed only a weak staining pattern. During subsequent IP studies, strong bands were precipitated by a small number of sera from blood-relatives (12; 5.6%), spouses (one; 4.2%), and normals (three; 2.5%), although none was identified as an SSc-specific autoantigen.

Thus, although antinucleolar reactivity was more common in the first-degree relatives of SSc patients than in normal controls, SSc-associated ANoAs were only present in individuals with the disease, and appeared to be mutually exclusive. Together, these results confirm the remarkably close association between the expression of certain defined ANoAs and the presence of SSc, while suggesting that a heritable factor may cause the nucleolus to become a focus of the immune response in both SSc patients and their blood-relatives. The autoantibodies detected in some healthy relatives of SSc patients may represent alternative responses to this shared susceptibility factor: the lack of certain other pro-pathological factors *in vivo* could have protected against disease onset, and may also have prevented the particular nucleolar antigen processing pathways which are required for the generation of SSc-specific ANoAs.

3.1 INTRODUCTION

The work described in this chapter concerns the initial detection and characterization of antinuclear and antinucleolar reactivities in the serum samples contributed by the U.K. SSc Study Group, *viz.* 58 SSc patients (the probands), four of their first-degree relatives with SSc, 215 of their first-degree relatives without SSc, and 24 spouses of the SSc patients. The directive was to screen all sera for anti-cytoplasmic, antinuclear and antinucleolar reactivities by IF. Each sample was also to be tested by immunodiffusion. All positive results were to be further investigated by IP using ^{35}S-methionine-labelled K562-cell extracts: defined ANA and ANoA specificities would then be identified, and any novel autoantibody systems would be further investigated. The disease subtype associations of the defined ANoAs detected in the patient group would also be examined.

In addition, the relative prevalences of antinuclear and antinucleolar reactivities in probands, relatives and spouses were compared, and sera from 118 randomly chosen CTD-free family members were separately compared with 120 age- and sex-matched blood-donor controls to determine whether they had an increased frequency of ANAs and/or ANoAs to that expected.

3.2 MATERIALS AND METHODS

Clinical details and serology

Clinical details were supplied for the 58 probands with SSc, 219 of their first-degree relatives and the 24 spouses (for complete details see Appendix IV), all of which were Caucasian. All families were British, apart from two Russian families (Families NRM and NRT), and three from the United States (Families NAA, NAB and NAC). Some families were selected on the basis of more than one member having a CTD – a total of four families had two members with a diagnosis of SSc (Families I, NAA, NRM and NRT). Serum samples from all probands and family members were supplied to our laboratory, and stored as described (Section 2.1.3).

Protocols regarding clinical examination, diagnosis and disease subtype assignation of patients and their family members are described in Section 2.1.2. All features characteristic of a CTD were recorded. All probands and four consanguineous relatives were confirmed as fulfilling the required criteria for SSc (218). Of the probands, 23 (39.7%) had dc-SSc, and 35 (60.3%) had lc-SSc. Most of the probands were female (91%). A further 12 consanguineous relatives were diagnosed as having a different CTD (two SLE, one SLE/Pm overlap, three RA, one morphea (and hyperthyroid), and one chronic active hepatitis); in addition, 62 relatives (26%) without SSc had symptoms of RP, three had PA, and two more had hyperthyroid. The 120 age- and sex-matched control sera were obtained from a blood-bank.

Immunofluorescence

All serum samples were screened by IF (Method 2.3.2) using (i) HEp-2 slides prepared in-house (Method 2.2.3), and (ii) commercially obtained HEp-2 slides (Biodiagnostics Ltd). Antinuclear and antinucleolar IF intensities were each scored as negative (-), very weak (+/-), weak (+), moderate (++), strong (+++) or very strong (++++). A score of ' -' was assigned only to those samples displaying minimal background staining (Fig. 3.1*a*; the samples shown in Figs 3.1*b* and 3.1*c* were assigned nuclear staining scores of '+/-' and '+', respectively, and are shown for comparison). Only those samples with a score of '+' and above were counted as positive. Cytoplasmic staining was also recorded, since such patterns are indicative of antibodies recognizing mitochondrial-E2, ribosomal RNP (rRNP), PL-7 and Jo-1 antigens, which are sometimes found to occur in SSc sera. A variety of prototype anti-cytoplasmic (Fig. 3.2*a-c*), antinuclear (Fig. 3.3*a,c,e,g*) and antinucleolar (Fig. 3.4*a,c,e,g,h*) sera with previously defined specificities were included as positive controls, and to aid with pattern identification.

In addition, all sera from family members who had SSc, SLE, Pm, morphea or PA were excluded before randomly selecting 105 sera from first-degree relatives (mean age 47.5 yr, S.D. 22.4 yr; range 7-88 yr; 49.5% male) and 13 sera from spouses (mean age 59.1 yr, S.D. 15.8 yr, range 40-87 yr; 91.7% male), which were intermingled with 120 normal control sera (mean age 50.9 yr, S.D. 12.3 yr, range 26-85 yr; 50.0% male). A double-blind study was then conducted, comparing antinuclear and antinucleolar IF intensity scores at a 1/40 dilution using slides from Biodiagnostics Ltd.

Immunodiffusion

Sera from all probands and family members were screened by immunodiffusion (Method 2.3.1) using RTE (Pel Freez Ltd), TIE (Bradsure Biologicals Ltd), RLE (Bradsure Biologicals Ltd) and CTE (in-house preparation) as the sources of antigens.

Radioimmunoprecipitation

For the identification of defined ANoAs, all samples showing any trace of nucleolar staining by IF (+/- or greater) were further tested by IP. Also, all patients with a defined CTD, and sera with moderate or strong nuclear staining by IF (at least ++), were tested by IP in order to detect any ANoAs missed by the other methods: a strong anti-nuclear pattern can mask a weaker anti-nucleolar reactivity occurring in the same serum, and sera with anti-RNAP antibodies in particular may recognize both nuclear and nucleolar constituents (Fig. 3.5*a*). All samples showing a positive result by immunodiffusion were also tested, as were those sera with distinct cytoplasmic patterns by IF.

Immunoprecipitation of ^{35}S-methionine-labelled K562-cell extracts was performed as described (Method 2.3.3*a*). Antinucleolar antibody specificities were identified by comparison with a set of reference antinucleolar sera (Pm-Scl, U3 RNP, Th RNP and RNAP I/II/III: kind gift from Dr Joe Craft, Yale University School of Medicine, U.S.A.; Fig. 3.6). Antinuclear specificities, including those detected by

immunodiffusion, were also identified, by comparison with prototype sera of defined autoantibody specificities from our own laboratory (topo I, Jo-1, PL-7, Ku, U1RNP±Sm, Ro, and La; Fig. 3.6).

3.3 RESULTS

Immunofluorescence

Although many ACAs, ANoAs and ANAs were detected using the in-house prepared HEp-2 slides, background fluorescence was a problem. It was for this reason that commercially available slides were sought: these were found to give a superior result, with less background staining and a generally cleaner appearance which was easier to read. Consequently, only those results obtained using commercial slides are reported and illustrated here.

Of the 62 sera from SSc patients (58 probands and four relatives), 61 (98.4%) had positive IF scores (centromere, nuclear and/or nucleolar). Thirteen sera (21.0%) showed the characteristic centromere staining pattern (Table 3.1; Fig. 3.3*f*), including four patients from two multicase families (samples NAA1 and 2, NRT1 and 2; (226)). Sixty-six (30.7%) of the 215 SSc-free relatives, and seven spouses (29.2%), had positive nuclear and/or nucleolar IF. None of the SSc-free family members showed any trace of ACAs.

Nuclear staining

Excluding sera displaying only nucleolar patterns, positive staining of the nucleus and/or nucleoplasm was found in 51 SSc patients (82.3%; Figs 3.2*d*, 3.3*b,d*, 3.4*b,f*, 3.5*a,b* and 3.7*b,c*), 50 SSc-free relatives (23.3%; Figs 3.3*i*, 3.8*c,g*) and six spouses (25.0%) (Table 3.2). These results were significantly different when comparing SSc patients with either SSc-free relatives or spouses (Table 3.2). Of the twelve family members with a moderate or stronger ANA (Table 3.3), one had SLE/Pm, one had RA, one had morphea, five had RP, and one had PA. Examples of cytoplasmic staining detected in SSc patients and SSc-free relatives can be seen in Figs 3.1*b*, 3.2*d*, 3.4*b,f*, and 3.7*b,d*, and in Fig. 3.8*b,f,g,h* respectively.

Nucleolar staining

Positive staining of the nucleolus was found when testing sera from 25 SSc patients (40.3%; Figs 3.2*d*, 3.3*b*, 3.4*b,d,f,h*, 3.5*b*, and 3.7*a,c,d*), 33 SSc-free relatives (15.3%; Figs 3.3*i* and 3.8*c,f,g,h*) and four spouses (16.7%) (Table 3.2), and these results were significantly different when comparing SSc patients with SSc-free relatives (Table 3.2). Moderate or stronger nucleolar staining was also significantly more frequent in sera from SSc patients (20 patients; 32.3%) than from SSc-free relatives (eleven; 5.1%), and

was absent in the case of spouses (Table 3.2). Of the eleven family members with a moderate or stronger ANoA (Table 3.3), three had RP and one had thyroid disease.

ANA and ANoA in relatives compared to controls

Anti-nucleolar IF was significantly more frequent in sera from CTD-free first-degree relatives than from age- and sex-matched blood-donor controls ($P = 0.029$; χ^2-test) (Table 3.4). As indicated, most of the difference between the two groups was accounted for at a weak level of reactivity (+). Nuclear IF was not significantly different between the three groups (Table 3.4).

Immunodiffusion

Nineteen sera from patients with SSc (30.6%) had precipitating antibodies by immunodiffusion (Tables 3.1 and 3.5; Fig. 3.9); sixteen of these were identified by this method (Fig 3.9), and the remainder were identified by subsequent IP. The antibodies identified in SSc patients by immunodiffusion recognized topo I (8), Pm-Scl (3), Jo-1 (2), U1 RNP (4), Sm (1), Ro (2) and La (1). No serum recognized both Pm-Scl and topo I, and no serum was found to contain both ACAs and anti-topo I antibodies.

None of the spouses, and only four SSc-free relatives (1.9%) had precipitating antibodies by immunodiffusion. Of these four relatives, one had SLE, one had RP, and one had PA. By immunodiffusion, both the SLE serum (BC4) and the PA serum (LJ8) had antibodies to Ro and La (Table 3.1; Fig. 3.9), results which were later confirmed by IP. The specificities of the other two sera (BE6 and LJ7) could not be identified (Table 3.6) by either immunodiffusion (Fig. 3.9) or subsequent IP (Fig. 3.11).

Radioimmunoprecipitation

Defined antinuclear specificities

All specificities determined by immunodiffusion were confirmed by IP, and several more were detected (Table 3.1). Although IP is not a suitable method for the detection of ACAs, all sera positive for ACAs by IF were tested for the presence of other specificities (Fig. 3.10), but none was detected. Excluding ACAs, a total of 30 sera (28 SSc, one SLE/Pm and one PA) had autoantibodies to a defined nuclear antigen (Ro (6 sera; Fig. 3.11), La (4 sera; Fig. 3.11), U1 RNP (5 sera; Fig. 3.11), Sm (one serum; Fig. 3.11), Ku (one serum; Fig. 3.12), Jo-1 (three sera; Fig. 3.11), PL-7 (1 serum), topo I (eight sera; Fig. 3.13*a*), RNAP II (nine sera; Figs 3.14 and 3.15), and RNAP III (eleven sera; Figs 3.14 and 3.15) (Table 3.1). A number of sera displayed multiple ANA specificities (Table 3.1), and will be discussed presently.

All sera found to contain ANAs of defined specificities had produced nuclear staining by IF (Table 3.1; Figs 3.3*b,d,i*, 3.5*b* and 3.7*c*), but five had also produced nucleolar staining of an even stronger intensity (Table 3.1): in two cases this was explained by the subsequent detection of defined ANoAs in these sera, however, three

ANA sera (all containing both anti-topo I and anti-Ro antibodies) had produced predominantly nucleolar staining patterns (Table 3.1; Fig. 3.5*b*). While topo I is known to be located in both the nucleus and nucleolus, this was an interesting result, especially when compared with the staining pattern produced by a typical anti-topo I serum (Fig. 3.3*g*).

Defined antinucleolar specificities

Of the 62 SSc patients, 70 SSc-free relatives and eight spouses whose sera were tested by IP, 24 were found to have autoantibodies to a defined nucleolar antigen (RNAP I (ten sera; Figs 3.14 and 3.15), Pm-Scl (seven sera; Figs 3.15 and 3.16), U3 RNP (four sera; Figs 3.15 and 3.17), and Th RNP (three sera; Fig. 3.15) (Table 3.5). All of these were from SSc patients (38.7%).

All sera with anti-U3 RNP specificities had produced a distinct clumpy nucleolar pattern on IF (Table 3.5; Fig. 3.4*h*). Anti-Th RNP and anti-Pm-Scl antibodies had also produced staining of the nucleolus, though this was occasionally obscured by additional nucleoplasmic staining (Table 3.5; Fig. 3.4*d* and *f*, respectively). All anti-RNAP I sera were also found to contain anti-RNAP III antibodies (Table 3.5), and all had produced positive staining of the nucleoplasm by IF (Table 3.5; Figs 3.4*b* and 3.5*a*): while RNAP I is a nucleolar enzyme, RNAPs II and III are located in the nucleoplasm.

Sera containing multiple defined specificities

Four sera were found to precipitate both topo I and the metabolically active, phosphorylated (IIO) form of RNAP II, and three of these also precipitated the inactive, unphosphorylated form of the enzyme (RNAP IIA) (Table 3.1; Figs 3.13*b* and 3.18). However, unlike anti-RNAP I and anti-RNAP III, anti-RNAP II antibodies are not specific for SSc, and have recently been reported in SLE and MCTD sera (297). Apart from sera with antibodies to both RNAP I and RNAP III (which are known to share structural homologies), only one serum was found to contain more than one SSc-specific antibody, *viz.* sample I1, which precipitated RNAPs I, IIO, IIA and III, as well as topo I (Table 3.1; Fig. 3.13*b*). Altogether 14 sera precipitated one or more RNAP complex. Antibodies to the three classes of RNAP in SSc, and their relationship to topo I antibodies, are investigated in detail in Chapter 4 (see also (128,299)). The three anti-topo I sera which also contained anti-Ro antibodies have already been mentioned (Table 3.1).

Undefined specificities

Twenty-nine of the immunoprecipitated samples (eight SSc patients (12.9%), 20 SSc-free relatives (9.3%) and one spouse (4.2%)) produced bands of unknown specificity, including samples from four probands that also had defined SSc-specific autoantibodies (Table 3.6; Figs 3.19 and 3.20). A band of ~115 kDa occurred in four SSc patients and seven SSc-free family members, including the spouse and three sisters of a proband

with anti-U1 RNP antibodies (Table 3.6; Fig. 3.20). However, many of the unidentified IP bands were weak, and strong unidentified bands were precipitated by sera from six SSc patients (9.7%), twelve SSc-free relatives (5.6%) and one spouse (4.2%) (Table 3.6).

A variety of staining patterns had been produced by these sera by IF (Table 3.6) including cytoplasmic, nuclear and nucleolar staining (Fig. 3.8), but no consistent pattern was produced by the eleven samples which had been found to precipitate a band of ~115 kDa (Table 3.6; Figs 3.7*b,c,d* and 3.8*b,c,f*)

Of the 32 normal sera selected for IP (by the same criteria), a smaller, though comparable, proportion also showed the presence of unidentified bands by IP (seven (5.8%); Fig. 3.21), though only three were strong (2.5%; Fig. 3.21), and none was found to precipitate a band of ~115 kDa.

Clinical associations

Associations between autoantibody specificities in sera from probands, and disease subtypes, are shown in Table 3.7. That the majority of ACAs occurred in patients with lc-SSc was consistent with previous reports, while the 50:50 distribution of anti-topo I antibodies between limited and diffuse disease was less typical (349). Both RNAP-II and RNAP-III antibodies were found to be more common in dc-SSc.

Clinical associations with each type of ANoA were consistent with other studies: antibodies to Pm-Scl and to Th RNP were associated with lc-SSc (251,252), while antibodies to U3 RNP and to RNAP I were associated with diffuse disease (254,278). In contrast to previous reports (278), however, no patients with antibodies to RNAP I had renal disease despite a minimum period of follow-up of five years (207). The lack of renal disease in the total proband group may reflect a bias in selection of suitable families to study, however (Dr N.J.McHugh, pers.comm.). There was no apparent association of ANoAs with RP in SSc-free relatives (data not shown).

TABLE 3.1 Sera from SSc patients and their relatives found to contain antinuclear antibodies of defined specificities. Specificities were identified by radio-immunoprecipitation unless otherwise stated

Sample	Sex	Clinical features	Relation-proband	Autoantibodies identified	Nuclear IF score[¶]	Nucleolar IF score[¶]
L1	F	lc-SSc	-	ACA[§]	++++cent.	-
MB1	F	dc-SSc	-	ACA[§]	++++cent.	-
MC1	F	dc-SSc	-	ACA[§]	++++cent.	-
NAA1	F	lc-SSc	-	ACA[§]	++++cent.	-
NAA2	F	lc-SSc	Sister	ACA[§]	+++cent.	-
NAB1	F	dc-SSc	-	ACA[§]	+++cent.	-
NAC1	F	dc-SSc	-	ACA[§]	++++cent.	-
NRT1	F	lc-SSc	-	ACA[§]	++++cent.	-
NRT2	F	lc-SSc	Id. twin	ACA[§]	+++cent.	-
O1	F	lc-SSc	-	ACA[§]	+cent.	-
P1	F	lc-SSc	-	ACA[§]	++cent.	-
SD1	F	lc-SSc	-	ACA[§]	++++cent.	-
X1	F	lc-SSc	-	ACA[§]	++++cent.	-
SF1	F	dc-SSc	-	RNAP III	+f.sp	+sp.
BE1	F	dc-SSc	-	RNAP I,III[#]	+++dis.sp.	+sp.
LC1	F	dc-SSc	-	RNAP I,III	++dif.gr.sp.	*
SH1	F	lc-SSc	-	RNAP I,III	+f.sp.	+sp.
U1	F	dc-SSc	-	RNAP I,III	+++h.	++h.
BG1	F	lc-SSc	-	RNAP I,IIO,IIA,III	+f.sp.	-
LJ1	F	dc-SSc	-	RNAP I,IIO,IIA,III	+++h./gr.sp.	*
N1	F	dc-SSc	-	RNAP I,IIO,IIA,III	+dis.sp.	-
SE1	F	dc-SSc	-	RNAP I,IIO,IIA,III	++++h./sp.	*
V1	F	dc-SSc	-	RNAP I,IIO,IIA,III	++f.sp.	++++sp.
I1	M	dc-SSc	-	RNAP I,IIO,IIA,III,topo I[†]	+++f.sp.	+++sp.
I3	F	dc-SSc	Daughter	RNAP IIO,IIA,topo I[†]	+++h.sp.	-
NRM2	F	lc-SSc	Daughter	RNAP IIO,IIA,topo I[†],Ro[†],La	++f.sp	+++sp.
LA1	M	lc-SSc, dLE	-	RNAP IIO,topo I[†]	++++h./f.sp.	-
LD1	F	dc-SSc, Mor	-	topo I[†]	+cs.sp.	-
MD1	F	lc-SSc	-	topo I[†]	+++h./sp.	+
MK1	M	dc-SSc	-	topo I[†],Ro[†],La[†]	++f.sp.	+++sp.
NRM1	F	lc-SSc	-	topo I[†],Ro	++dif.f.sp.	+++h.
BB1	F	dc-SSc	-	Jo-1[#]	++gr.sp.	+
MI1	F	lc-SSc	-	Jo-1[†]	+++sp.	-
MM1	F	lc-SSc	-	Ku	++++sp.	-
G1	F	lc-SSc	-	PL-7, Pm-Scl	++f.sp.	+++h.
BC4	F	SLE/Pm	Mother	Ro[†], La[†]	++f.sp.	-
LJ8	M	PA	Father	Ro[†], La[†]	++++sp.	+
LK1	F	lc-SSc	-	Ro	+++dis.cs.sp.	-
BC1	F	lc-SSc	-	U1 RNP[†],Sm[†], Jo-1[†]	++++f.sp.	-
BD1	F	dc-SSc	-	U1 RNP[†]	++++cs.dif.sp.	-
LG1	F	lc-SSc	-	U1 RNP[†]	++++cs.sp.	-
MN1	F	lc-SSc	-	U1 RNP[†]	++++dif.cs.sp.	-
SG1	F	dc-SSc	-	U1 RNP±Sm	+f.sp.	-

*The staining pattern of the nucleolus was not distinct from the nucleoplasmic pattern.
[†]Identified by immunodiffusion
[§]Identified by indirect immunofluorescence (IF)
[¶]for IF scoring system definitions, see text
[#]Precipitin detected but not identified by immunodiffusion
lc-SSc, limited cutaneous SSc; dc-SSc, diffuse cutaneous SSc; dLE, discoid lupus erythematosus; Mor, morphea; SLE, systemic lupus erythematosus; Pm, polymyositis; PA, pernicious anaemia; ACA, anti-centromere antibody; RNAP, RNA polymerase; topo I, topoisomerase I; cent. centromere; f. fine; dif. diffuse; dis. discrete; cl. clumpy; gr. grainy; sp. speckled; h. homogeneous; cs. course

TABLE 3.2 Comparison between indirect immunofluorescence scores of sera from SSc patients and from their SSc-free family members

		Nuclear staining						Nucleolar staining					
		Patients (n=62)		Relatives (n=215)		Spouses (n=24)		Patients (n=62)		Relatives (n=215)		Spouses (n=24)	
Immunofluorescence score													
Negative	-	9	(14.5)	111	(51.6)	13	(54.2)	37	(59.7)	158	(73.5)	16	(66.7)
	±	2	(3.2)	54	(25.1)	5	(20.8)	0	(0.0)	24	(11.2)	4	(16.7)
Positive*	+	12	(19.4)	39	(18.1)	5	(20.8)	5	(8.1)	22	(10.2)	4	(16.7)
	++	10	(16.1)	6	(2.8)	1	(4.2)	3	(4.8)	7	(3.3)	0	(0.0)
	+++	14	(22.6)	3	(1.4)	0	(0.0)	9	(14.5)	3	(1.4)	0	(0.0)
	++++	15	(24.2)	2	(0.9)	0	(0.0)	8	(12.9)	1	(0.5)	0	(0.0)
	Total positive (+)*	**51**	**(82.3)§**	**50**	**(23.3)**	**6**	**(25.0)**	**25**	**(40.3)¶**	**33**	**(15.3)**	**4**	**(16.7)**
	Total positive (++)†	**39**	**(62.9)**	**11**	**(5.1)**	**1**	**(4.2)**	**20**	**(32.3)#**	**11**	**(5.2)**	**0**	**(0.0)**

Figures in parentheses are percentages

*Positive sera had a score of at least "+"

†Number of sera positive when using "++" as cut-off point for positivity

§$P < 0.0001$ compared with either relatives or spouses (χ^2-test)

¶$P < 0.0001$ compared with relatives (χ^2-test); not significant compared with spouses, but note low titre in the four spouses: on using ++ as cut-off point for positivity# ANoAs are significantly more frequent in patients than in relatives ($P < 0.0001$) or spouses ($P < 0.005$) (χ^2-test)

TABLE 3.3 Clinical status of SSc-free family members whose sera produced moderate (++) antinuclear and/or antinucleolar staining by indirect immunofluorescence

Sample	Clinical features	Relation to proband	Nuclear IF score¶	Nucleolar IF score¶	Unidentified bands by IP (kDa)	Identified auto-antibodies
BA2	-	Sister	-	++++h.	90/92/95	-
BC4	SLE/Pm	Mother	++f.sp.	-	-	Ro†, La†
BC6	Arthritis	Sister	++dis.cs.sp.	+/-	-	-
BD3	RP	Sister	+++f.sp.	+/-	115	-
BD5	RP	Sister	+++g.sp.	+sp.	115	-
BE6	RP	Mother	+/-g.sp.	+++sp.	115#	-
BG3	-	Husband	++f.sp.	-	-	-
F2	RP	Mother	+++cs.sp.	+++sp.	15 (wk)	-
LJ8	PA	Father	++++sp.	+	-	Ro†, La†
MC2	-	Daughter	+/-f.sp.	++cl.	-	-
MD3	-	Sister	+sp.	++sp./cl.	-	-
MD5	RP	Brother	++sp.	++sp./cl.	-	-
MD9	-	Son	+sp.	+++sp.	115	-
MI2	-	Sister	++cs.sp.	-	-	-
MO2	-	Son	+sp.	++sp.	-	-
NA3	Morphea	Sister	++++dis.sp.	-	-	-
NR2	-	Sister	+sp.	++sp.	-	-
O5	-	Father	-	++sp./cl.	49	-
SC6	-	Grandfather	++cs.sp.	+sp.	37 (wk)	-
SG2	RP	Mother	++sp.	-	-	-
V8	Hyperthyroid	Sister	-	++h.	-	-

†Identified by immunodiffusion
¶for indirect immunofluorescence (IF) scoring system, see text
#Precipitin detected but not identified by immunodiffusion
IP, immunoprecipitation; SLE, systemic lupus erythematosus; Pm, polymyositis; RP, Raynaud's phenomenon; PA, pernicious anaemia; f, fine; sp, speckled; dis, discrete; cs, course; g, grainy; h, homogeneous; cl, clumpy.

TABLE 3.4 Comparison between indirect immunofluorescence scores of sera from randomly selected connective tissue disease-free family members and normal controls

Immunofluorescence score		Nuclear staining						Nucleolar staining					
		SSc relatives (n=105)		Spouses (n=13)		Normal controls (n=120)		SSc relatives (n=105)		Spouses (n=13)		Normal controls (n=120)	
Negative	-	66	(62.9)	10	(76.9)	82	(68.3)	77	(73.3)	9	(69.2)	101	(84.2)
	±	21	(20.0)	3	(23.1)	23	(19.2)	9	(8.6)	3	(23.1)	9	(7.5)
Positive*	+	11	(10.5)	0	(0.0)	9	(7.5)	13	(12.4)	1	(7.7)	4	(3.3)
	++	6	(5.7)	0	(0.0)	6	(5.0)	5	(4.8)	0	(0.0)	6	(5.0)
	+++	1	(1.0)	0	(0.0)	0	(0.0)	1	(1.0)	0	(0.0)	0	(0.0)
	++++	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Total positive (+)*		**18**	**(17.1)**	**0**	**(0.0)**	**15**	**(12.5)**	**19§**	**(18.1)**	**1**	**(7.7)**	**10**	**(8.3)**
Total positive (++)†		**7**	**(6.7)**	**0**	**(0.0)**	**6**	**(5.0)**	**6**	**(5.7)**	**0**	**(0.0)**	**6**	**(5.0)**

Figures in parentheses are percentages
*Positive sera had a score of at least "+"
†Number of sera positive when using "++" as cut-off point for positivity
§$P < 0.05$ compared with normal controls (χ^2-test).

TABLE 3.5 Sera from SSc patients found to contain antinucleolar antibodies of defined specificities. Antinucleolar antibody specificities were identified by radio-immunoprecipitation

Patient	Sex	Clinical features	Autoantibodies detected by IP	Nuclear IF score	Nucleolar IF score
BA1	F	dc-SSc	Pm-Scl†	+ f.sp.	++h.
E1	F	dc-SSc	Pm-Scl#	Neg.	+++cl./sp
G1	F	lc-SSc	Pm-Scl†, PL-7	++f.sp.	+++h.
LE1	F	lc-SSc	Pm-Scl†	Neg.	+++h.
MP1	F	lc-SSc	Pm-Scl	Neg.	+++cl.
NA1	F	lc-SSc	Pm-Scl#	++f.sp.	++++sp.
SC1	F	lc-SSc	Pm-Scl	+++h.	*
BE1	F	dc-SSc	RNAP I,III#	+++dis.sp.	+sp.
BG1	F	lc-SSc	RNAP I,II,III	+f.sp.	*
I1	M	dc-SSc	RNAP I,II,III,topo I†	+++f.sp.	+++sp.
LC1	F	dc-SSc	RNAP I,III	++dif.gr.sp.	*
LJ1	F	dc-SSc	RNAP I,II,III	+++h./gr.sp.	*
N1	F	dc-SSc	RNAP I,II,III	+dis.sp.	*
SE1	F	dc-SSc	RNAP I,II,III	++++h./sp.	*
SH1	F	lc-SSc	RNAP I,III	+f.sp.	+sp.
U1	F	dc-SSc	RNAP I,III	+++h.	++h.
V1	F	dc-SSc	RNAP I,II,III	++f.sp.	++++sp.
LL1	F	lc-SSc, arthritis	Th RNP	+++h./sp.	*
MA1	F	lc-SSc	Th RNP	+/-f.sp.	++++sp.
SI1	F	lc-SSc	Th RNP	+sp.	+++cl.
B1	F	lc-SSc	U3 RNP	Neg.	++++cl./sp.
SA1	F	dc-SSc	U3 RNP	Neg.	++++cl.
T1	F	dc-SSc	U3 RNP	Neg.	++++cl.
W1	F	dc-SSc	U3 RNP	Neg.	++++cl.

*The staining pattern of the nucleolus was not distinct from the nucleoplasmic pattern.
†Also identified by immunodiffusion
¶for indirect immunofluorescence (IF) scoring system, see text
#Precipitin detected but not identified by immunodiffusion
IP, immunoprecipitation; lc-SSc, limited cutaneous SSc; dc-SSc, diffuse cutaneous SSc; RNAP, RNA polymerase; topo I, topoisomerase I; h. homogeneous; cl. clumpy; sp. speckled; f. fine; dif. diffuse; gr. grainy; dis. discrete.

TABLE 3.6 Sera from SSc patients and their family members found to precipitate unidentified autoantigens by radioimmunoprecipitation

Sample	Sex	Clinical features	Relation to proband	Unidentified bands by IP (kDa)	Identified auto-antibodies	Nuclear IF score¶	Nucleolar IF score¶
A1	F	lc-SSc	-	140	-	-	++++h.
A2	M	-	Father	105 (wk)	-	-	+h.
B1	F	lc-SSc	-	49,57,115,190	U3 RNP	-	++++cl./sp.
B8	F	-	Sister	18 (wk)	-	+cs.sp.	+h.
BA2	F	-	Sister	90,92,95	-	-	++++h.
BA3	F	-	Twin sister	65	-	+/-	+/-
BB2	F	-	Sister	35 (wk)	-	+/-f.sp.	+h.
BD2	M	-	Husband	115	-	+f.sp.	+sp.
BD3	F	RP	Sister	115	-	+++f.sp.	+/-
BD5	F	RP	Sister	115	-	+++gr.sp.	+sp.
BD7	F	-	Sister	115	-	-	-
BE6	F	RP	Mother	115#	-	+/-gr.sp	+++sp.
E6	F	-	Sister	130 (wk)	-	-	+/-
F2	F	RP	Mother	15 (wk)	-	+++cs.sp.	+++sp.
I4	F	-	G.daughter	28,30	-	+/-cs.sp.	-
K1	F	lc-SSc	-	115 (wk)	-	++cs.sp.	-
LA6	M	-	Father	190 (wk)	-	+sp.	+sp.
LJ7	F	-	Mother	115#	-	+f.sp.	-
LK1	F	lc-SSc	-	115	Ro	+++dis.cs.sp.	-
MD9	M	-	Son	115	-	+sp.	+++sp.
MJ2	M	-	Son	17 (wk)	-	-	+sp.
MO1	F	lc-SSc	-	20 (wk)	-	-	++cl.
NR1	F	SSc	-	25	-	+gr.sp.	-
NRM1	F	lc-SSc	-	115,125	topo I†,Ro	++dif.f.sp.	+++h.
NRM7	M	RP	Son	25 (wk)	-	+/-sp.	+/-
O5	M	-	Father	49	-	-	++sp./cl.
SC1	F	lc-SSc	-	240	Pm-Scl	+++h.	*
SC3	F	-	Sister	37 (wk)	-	+/-gr.sp.	+/-
SC6	M	-	G.father	37 (wk)	-	++cs.sp.	+sp.

*The staining pattern of the nucleolus was not distinct from the nucleoplasmic pattern
†Identified by immunodiffusion
¶for indirect immunofluorescence (IF) scoring system, see text
#Precipitin detected but not identified by immunodiffusion
IP, immunoprecipitation; lc-SSc, limited systemic sclerosis; RP, Raynaud's phenomenon; wk weak; cs. course, sp. speckled; f. fine; gr. grainy; dis. discreet; dif. diffuse; h. homogeneous; cl. clumpy

TABLE 3.7 Disease subtypes of probands with defined autoantibody specificities: (*a*) antinuclear antibody specificities; (*b*) antinucleolar antibody specificities

(*a*)

		Disease subtype	
	No. of probands	lc-SSc *n*=35 (60.3)	dc-SSc *n*=23 (39.7)
Antinuclear antibody			
ACA	11 (19.0)	7 (63.6)	4 (36.4)
Jo-1	3 (5.2)	2 (66.7)	1 (33.3)
Ku	1 (1.7)	1 (100.0)	0 (0.0)
PL-7	1 (1.7)	1 (100.0)	0 (0.0)
RNAP IIO±IIA	7 (12.1)	2 (28.6)	5 (71.4)
RNAP III	11 (19.0)	2 (18.2)	9 (81.8)
Ro±La	3 (5.2)	2 (66.7)	1 (33.3)
Topo I	6 (10.3)	3 (50.0)	3 (50.0)
U1 RNP±Sm	5 (8.6)	3 (60.0)	2 (40.0)
Total positive:	**37* (63.8)**		

(*b*)

		Disease subtype	
	No. of probands	lc-SSc *n*=35 (60.3)	dc-SSc *n*=23 (39.7)
Antinucleolar antibody			
Pm-Scl	7 (12.1)	5 (71.4)	2 (28.6)
RNAP I	10 (17.2)	2 (20.0)	8 (80.0)
Th RNP	3 (5.2)	3 (100.0)	0 (0.0)
U3 RNP	4 (6.9)	1 (25.0)	3 (75.0)
Total positive:	**24 (41.4)**		

Figures in parentheses are percentages.
*ten sera had more than one of these antinuclear antibody specificities
ACA, anti-centromere antibody; RNAP, RNA polymerase; topo I, topoisomerase I; lc-SSc, limited cutaneous systemic sclerosis; dc-SSc, diffuse cutaneous SSc

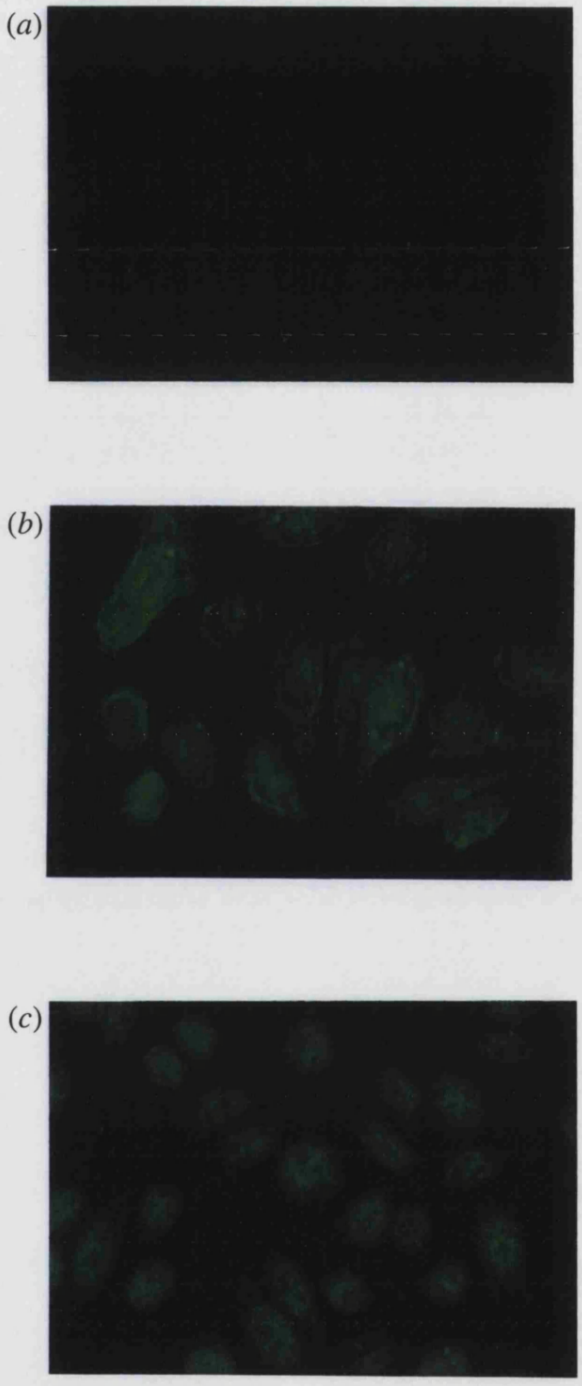


FIGURE 3.1 Indirect immunofluorescence scoring system. (*a*) Negative (-) staining pattern produced by a prototype normal human serum taken from a healthy donor. (*b*) Serum from proband SB1 had a negative nucleolar score and barely discernible (+/-) nuclear staining, which was counted as negative; stronger cytoplasmic staining was also evident, but no autoantibodies were subsequently identified. (*c*) Control serum NS27, from a healthy donor, produced weak, diffuse fine speckled nuclear staining (+), sparing the nucleolus. However, no autoantibodies were detected by other serological tests.

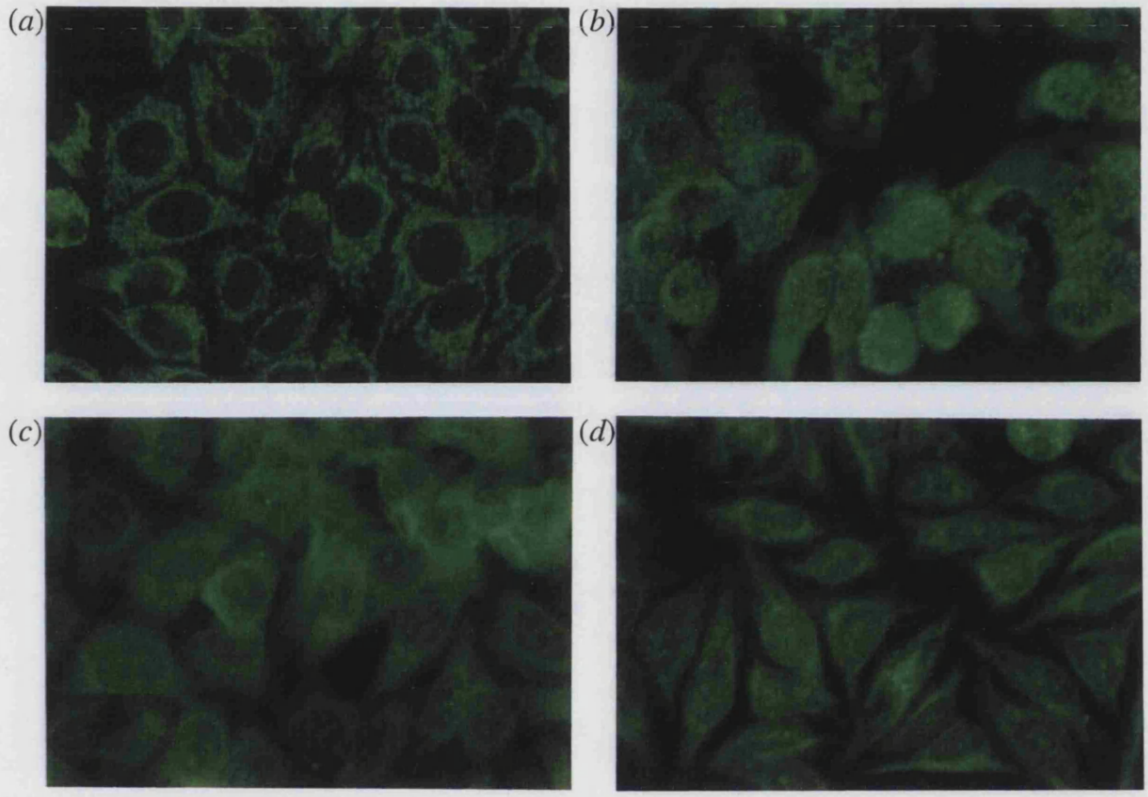


FIGURE 3.2 Indirect immunofluorescence: cytoplasmic patterns produced by sera with defined autoantibody specificities from patients with connective tissue diseases. (*a*) Course speckled cytoplasmic pattern produced by a prototype anti-mitochondrial-E2 serum from a patient with primary biliary cirrhosis. (*b*) Fine speckled cytoplasmic pattern produced by a prototype anti-ribosomal RNP (rRNP) serum from an SLE patient; speckled staining of the nucleus was also apparent (*c*) Very fine speckled cytoplasmic pattern produced by a prototype anti-PL-7 serum from an SSc patient. (*d*) Serum from proband BB1 produced a very fine, filamentous cytoplasmic pattern along with a weak grainy nuclear speckle; nucleoli can also be distinguished in some cells: anti-Jo-1 antibodies were subsequently identified by immunodiffusion.

FIGURE 3.3 **Indirect immunofluorescence: nuclear patterns produced by sera with defined autoantibody specificities from patients with connective tissue diseases (CTDs).** (*a*) Homogeneous nuclear staining produced by a prototype anti-DNA serum from a systemic lupus erythematosus (SLE) patient. (*b*) Serum sample SF1 apparently produced a weak homogeneous pattern; however, on close inspection, this was seen to consist of dense fine speckles, and some nucleoli were distinguishable: anti-RNA polymerase III antibodies were subsequently identified by radioimmunoprecipitation techniques (Fig. 3.13*b*, lane 2). (*c*) Diffuse, fine speckled nuclear staining, nucleolar sparing, produced by a prototype anti-Ro serum from an SLE patient. (*d*) Diffuse, course speckled nuclear staining, nucleolar sparing, was produced by sample BD1: anti-U1 RNP antibodies were subsequently identified by immunodiffusion (Fig. 3.11, lane 5). (*e*) Discrete course speckled nuclear pattern produced by a prototype anti-centromere serum from an SSc patient. (*f*) A strong, characteristic centromere pattern was also produced by serum sample NAC1. (*g*) Typical diffuse grainy speckled nuclear pattern with prominent nucleolar staining produced by a prototype anti-topoisomerase I serum from an SSc patient. (*h*) Diffuse, fine speckled/grainy nuclear pattern with nucleolar staining produced by a prototype anti-Ku serum from an SSc patient. (*i*) A diffuse, fine speckled nuclear pattern with occasional nucleolar staining was produced by serum LJ8, from a family member with pernicious anaemia: anti-Ro and anti-La antibodies were subsequently identified by immunodiffusion and confirmed by radioimmunoprecipitation (Fig. 3.13, lane 9). *(Continued overleaf...)*

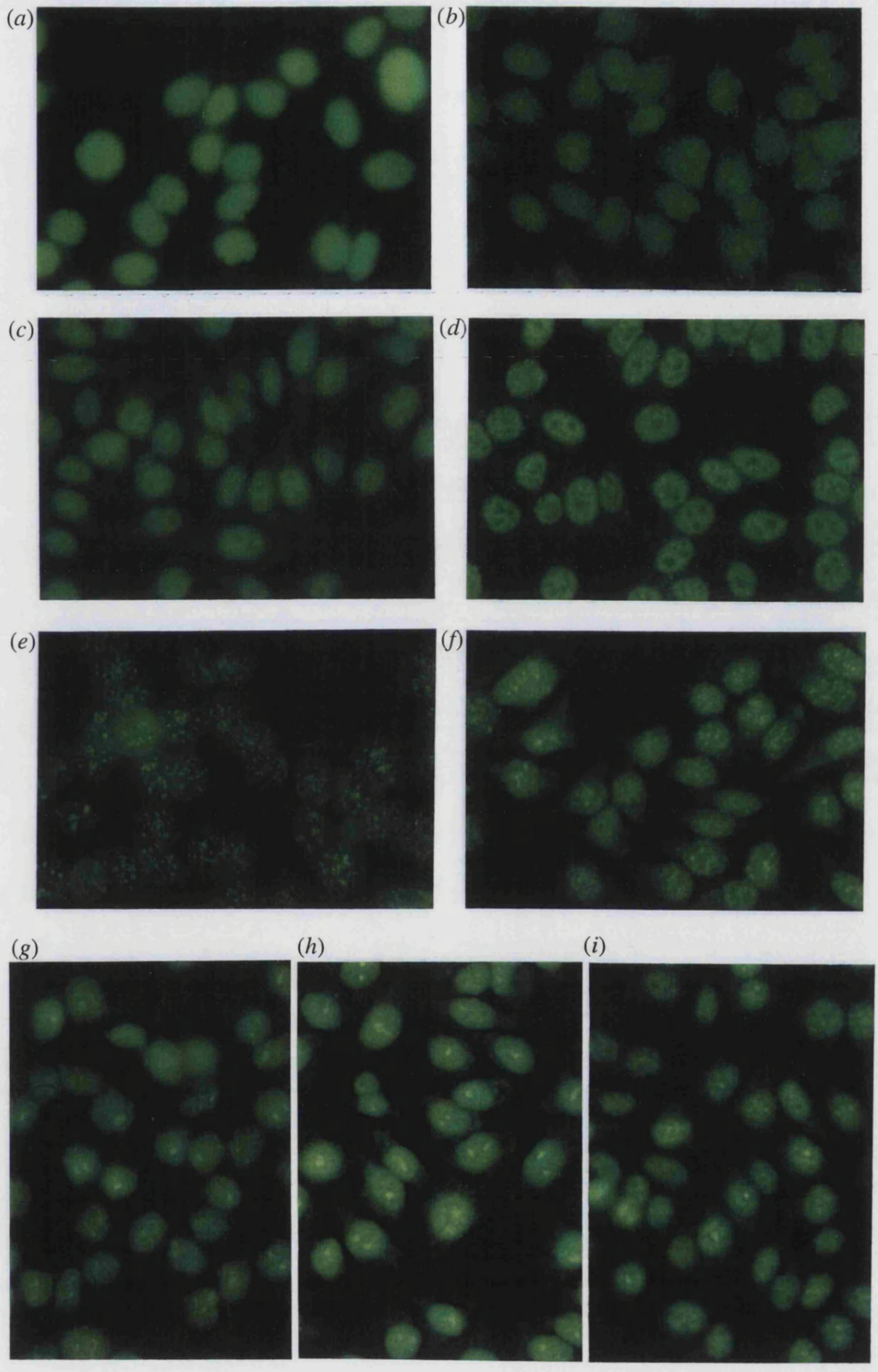


FIGURE 3.3 *(Continued)* **Indirect immunofluorescence: nuclear patterns produced by sera with defined autoantibody specificities from patients with CTDs.**

FIGURE 3.4 Indirect immunofluorescence: nucleolar patterns produced by sera with defined autoantibody specificities from patients with SSc (*a*) Weak fine speckled nuclear staining with a speckled nucleolar pattern was produced by a prototype anti-RNA polymerase (RNAP) I/III serum from an SSc patient. (*b*) Fine speckled nuclear staining with a strong speckled nucleolar pattern was produced by serum V1, and very weak cytoplasmic staining was also seen: anti-RNAP I/II/III antibodies were subsequently identified by radioimmunoprecipitation (Fig. 3.14*a*, lane 5).(*c*) A speckled nucleolar pattern in the absence of nucleoplasmic staining was produced by a prototype anti-Th RNP serum. (*d*) A very strong speckled nucleolar pattern with feint speckled nuclear staining produced by serum MA1: anti-Th RNP antibodies were subsequently identified by radioimmunoprecipitation techniques (Fig. 3.15*b*, lane 7). (*e*) Homogeneous nucleolar pattern with weaker, fine speckled nuclear staining produced by a prototype anti-Pm-Scl serum. (*f*) A homogeneous nucleolar pattern with fine speckled nuclear staining was produced by serum BA1, and weak grainy cytoplasmic staining was also visible: anti-Pm-Scl antibodies were subsequently identified by radioimmunoprecipitation techniques (Fig. 3.16, lane 5). (*g*) Characteristic clumpy nucleolar staining produced by a prototype anti-U3 RNP serum. (*h*) Strong clumpy nucleolar staining was produced by serum W1: anti-U3 RNP antibodies were subsequently identified by radioimmunoprecipitation techniques (Fig. 3.15*a*, lane 5). *(Continued overleaf...)*

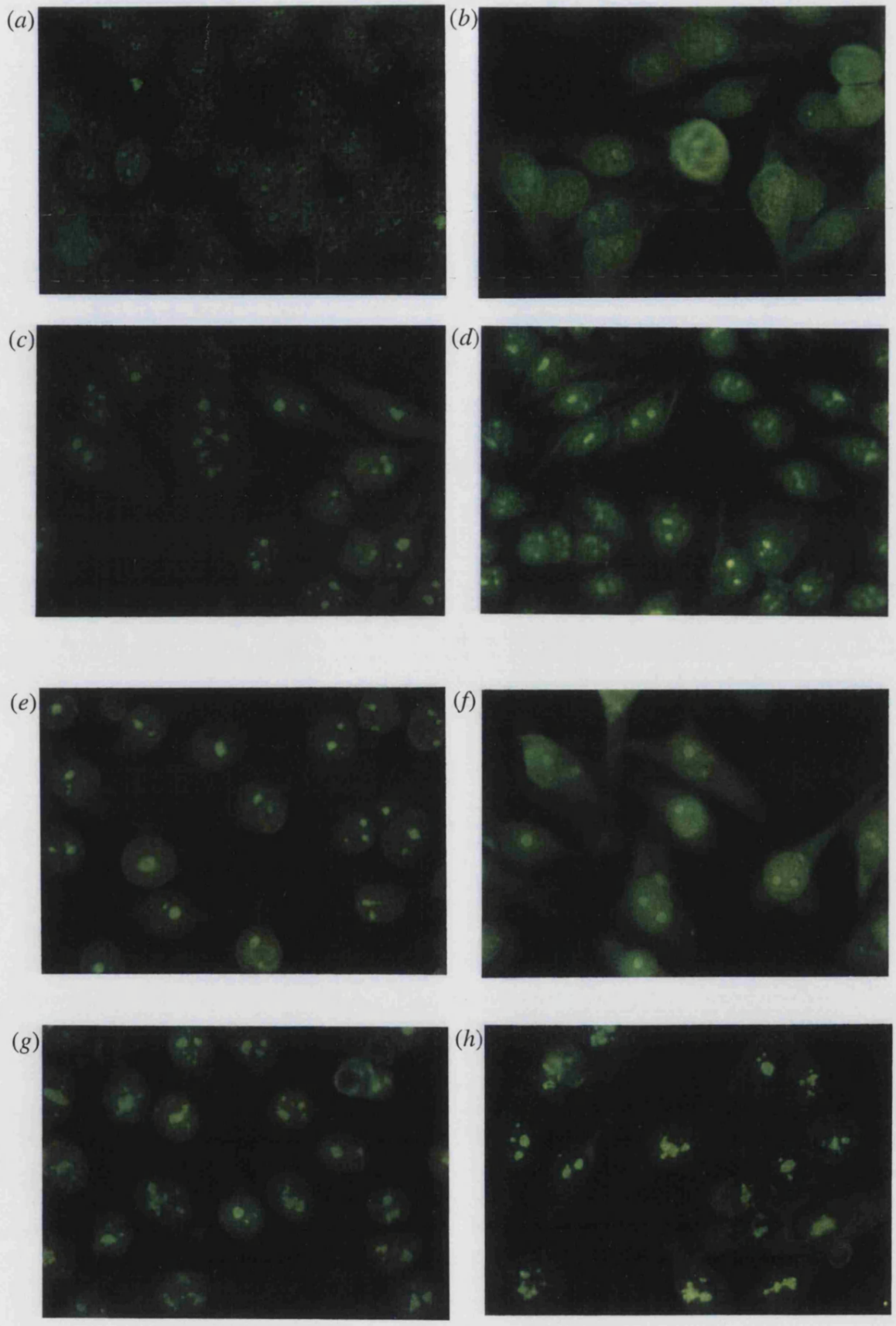


FIGURE 3.4*(Continued)* Indirect immunofluorescence: nucleolar patterns produced by sera with defined autoantibody specificities from patients with SSc.

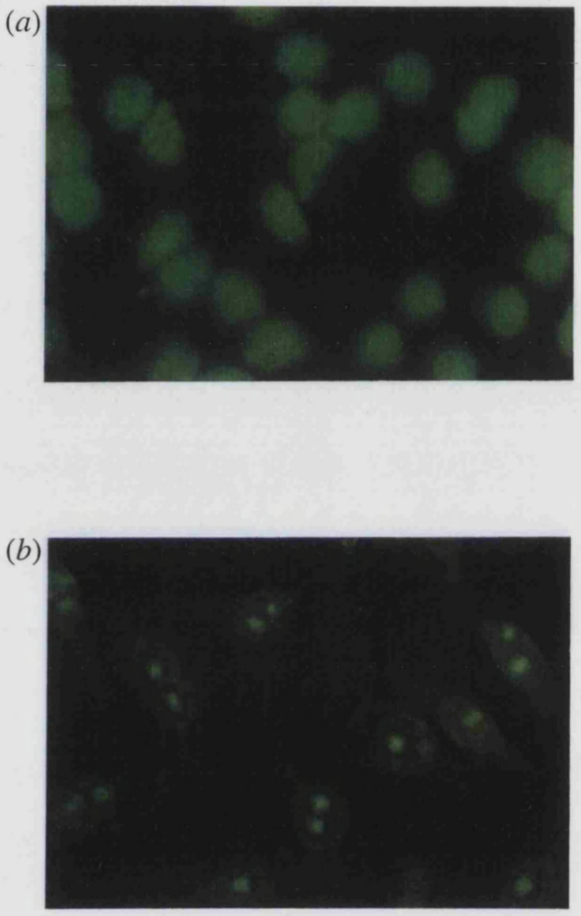


FIGURE 3.5 Indirect immunofluorescence: interpretation and significance of staining patterns produced by sera from patients with connective tissue diseases (*a*) A homogeneous/grainy speckled nuclear pattern was produced by serum LJ1. Anti-RNA polymerase (RNAP) I/II/III antibodies were subsequently identified by radio-immunoprecipitation techniques (Fig. 3.15*a*, lane 8). Although RNAP I is a nucleolar antigen, nucleolar staining had apparently been obscured by strong nuclear staining produced by anti-RNAP II and III antibodies. (*b*) Serum MK1 appeared to contain predominantly anti-nucleolar antibodies, with only weak anti-nuclear staining, but only antinuclear antibodies were subsequently identified (Ro, La and topoisomerase I; Fig. 3.13*a*, lane 10). While the La and topoisomerase I antigens are nuclear proteins they are also found in the nucleolus.

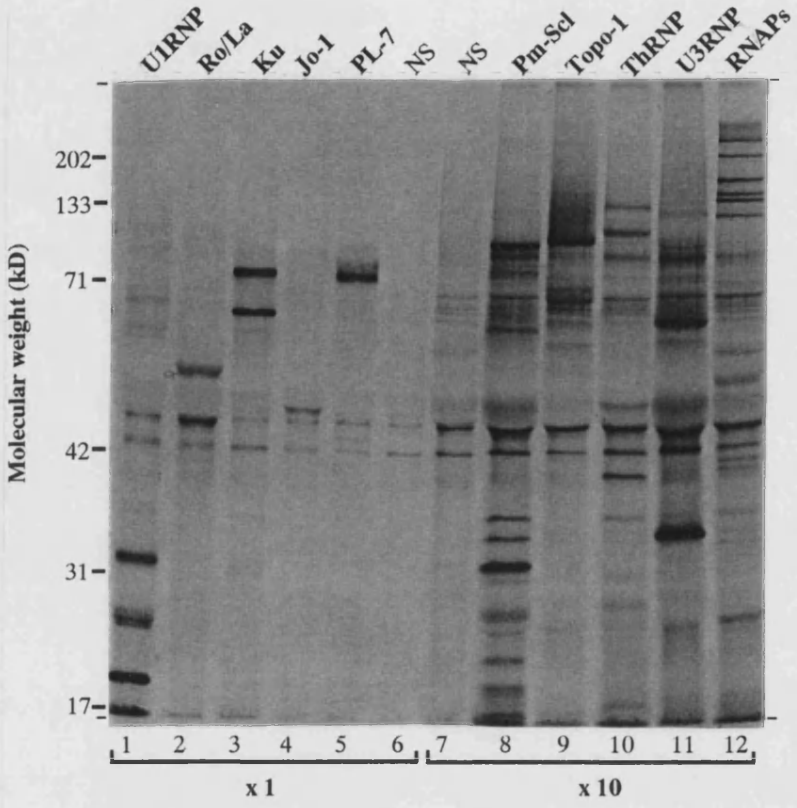


FIGURE 3.6 Radioimmunoprecipitation of prototype sera with defined antinuclear and antinucleolar antibody specificities. Autoradiograph of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. Samples of immuno-precipitated proteins were diluted in sample buffer x10 before loading into lanes 1-6 (lane 1, anti-U1 RNP standard; lane 2, anti-Ro/La standard; lane 3, anti-Ku standard; lane 4, anti-Jo-1 standard; lane 5, anti-PL-7 standard, lane 6, normal serum). Undiluted radioimmunoprecipitation (IP) samples were loaded into lanes 7-12. (lane 7, normal serum; lane 8, anti-Pm-Scl standard; lane 9, anti-topoisomerase I standard; lane 10, anti-Th RNP standard; lane 11, anti-U3 RNP standard; lane 12, anti-RNA polymerase I/II/III standard). Samples were prepared identically in all other respects, using prototype sera of high autoantibody titre. The technique of IP is particularly suitable for the identification of antibodies which recognize multimeric proteins, as a characteristic pattern of bands is produced (lanes 1, 3, 8, 10 and 12). However, the presence of antibodies which recognize monomeric proteins (lanes 2, 4, 5 and 9) can be confirmed by comparison with internal standards. The antigens detected in lanes 1-5 are relatively more abundant in K562-cell extracts than those in lanes 8-12, which are consequently more difficult to detect.

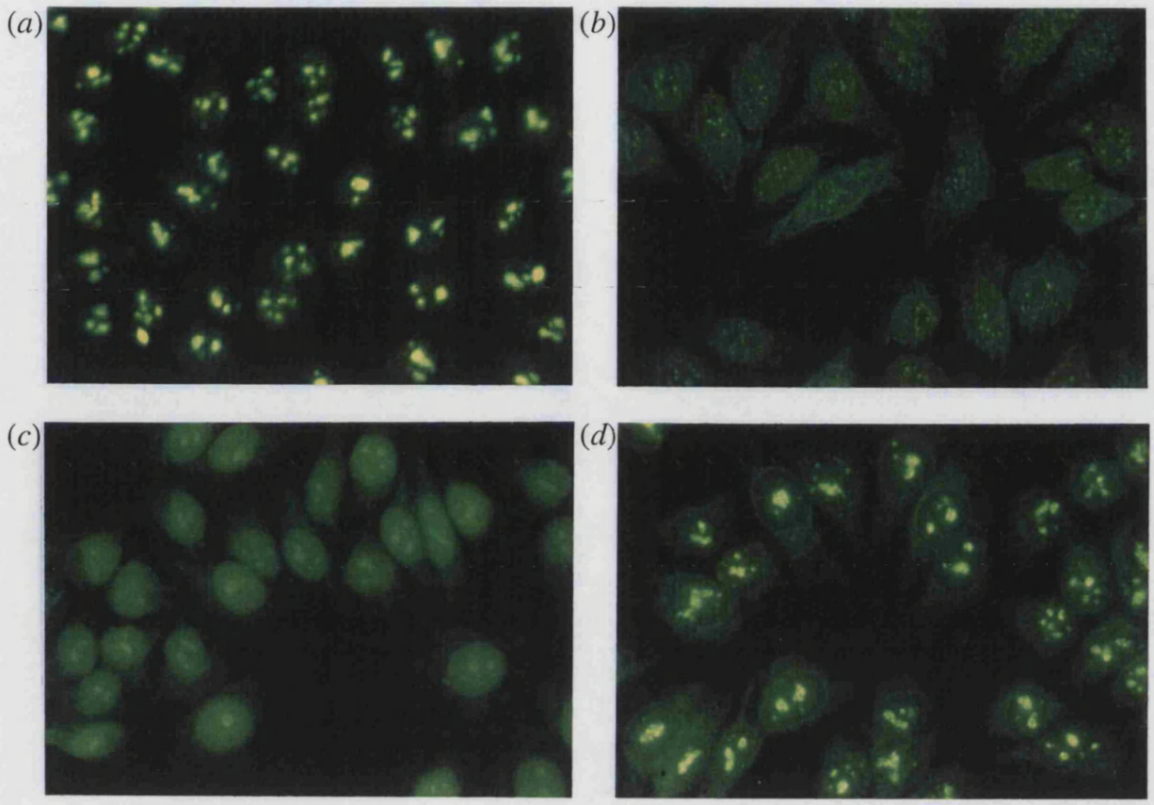


FIGURE 3.7 Indirect immunofluorescence patterns produced by sera from SSc patients which precipitated unidentified autoantigens by radioimmunoprecipitation. (*a*) A very strong homogeneous nucleolar pattern was produced by serum A1. Subsequent serological tests failed to identify antibodies of defined specificity: however, a strong, unidentified 140-kDa band was apparent by radioimmunoprecipitation (IP). (Fig 3.19, lane 11). (*b*) A discrete course speckled nuclear pattern was produced by serum K1, which was distinct from the characteristic anti-centromere pattern, and weak cytoplasmic filaments were also visible. Although no antibodies of defined specificity were detected by subsequent serological tests, a weak 115-kDa band was apparent by IP. (*c*) A diffuse fine speckled nuclear pattern with prominent nucleolar staining was produced by serum NRM1: anti-topoisomerase I antibodies were subsequently identified by immunodiffusion. A 100-kDa band was produced by IP, consistent with the presence of anti-topoisomerase I, and anti-Ro antibodies were also detected by IP (Fig. 3.13*a*, lane 5). In addition, unidentified bands of 115- and 125-kDa were produced on IP gels (Fig. 3.19, lane 2). (*d*) Clumpy/speckled nucleolar staining was produced by serum B1: anti-U3 RNP antibodies were subsequently identified by IP techniques (Fig. 3.17, lane 5). Unusually for a serum of this specificity (*cf.* Fig. 3.4*g* and *h*), fine speckled cytoplasmic staining, and perinuclear staining were also produced, and additional unidentified bands were apparent by IP (Fig 3.19, lane 13).

FIGURE 3.8 **Indirect immunofluorescence: nuclear and nucleolar patterns produced by sera from connective tissue disease (CTD)-free relatives which precipitated unidentified autoantigens by radioimmunoprecipitation.** (*a*) A very weak speckled nuclear and nucleolar pattern was produced by serum BA3, the healthy twin sister of proband BA1: no defined autoantibodies were detected by subsequent serological tests, but an unidentified 65-kDa band was produced by radioimmunoprecipitation (IP) (Fig. 3.19, lane 8). (*b*) Weak, fine speckled nuclear staining, nucleolar sparing, with weak speckled cytoplasmic staining was produced by serum from LJ7, the disease-free mother of SSc patient LJ1. Subsequent serological tests failed to identify antibodies of defined specificity: however, a strong, unidentified 115-kDa band was apparent by IP (Fig. 3.20, lane 9). (*c*) A strong diffuse grainy speckled nuclear pattern with very weak speckled nucleolar staining was produced by serum from BD5, a disease-free sister of SSc patient BD1. Although no antibodies of defined specificity were detected by subsequent serological tests, a very strong, unidentified 115-kDa band was apparent by IP (Fig. 3.20, Lane 6). (*d*) A very weak nucleolar pattern was produced by serum from E6, the disease-free sister of SSc patient E1. Subsequent serological tests failed to identify antibodies of defined specificity: however, a weak, unidentified band of 130 kDa was apparent by IP (Fig. 3.19, lane 9). (*e*) Weak nuclear and nucleolar patterns were produced by serum LA6, the disease-free father of SSc patient LA1. Although no antibodies of defined specificity were detected by subsequent serological tests, a weak unidentified 190-kDa band was apparent by IP (Fig. 3.19, lane 10). (*f*) A strong nucleolar pattern was produced by serum BE6 with very weak background nuclear staining and weak cytoplasmic staining; this serum also produced an unidentified precipitin by immunodiffusion, and an unidentified 115-kDa band by IP. (*g*) A strong nucleolar pattern with weak speckled nuclear staining and discernible cytoplasmic fluorescence was produced by serum from MD9, the disease-free son of SSc patient MD1. Subsequent serological tests failed to identify antibodies of defined specificity: however, a strong, unidentified band of 115 kDa was apparent by IP (Fig. 3.20, lane 5). (*h*) A strong nucleolar pattern was produced by serum from BA2, the disease-free sister of SSc patient BA1; peri-nuclear and weak cytoplasmic staining is also apparent. Although no antibodies of defined specificity were detected by subsequent serological tests, an unidentified triplet of (90-95 kDa) was produced by IP (Fig. 3.18, lane 7). *(Continued overleaf...)*

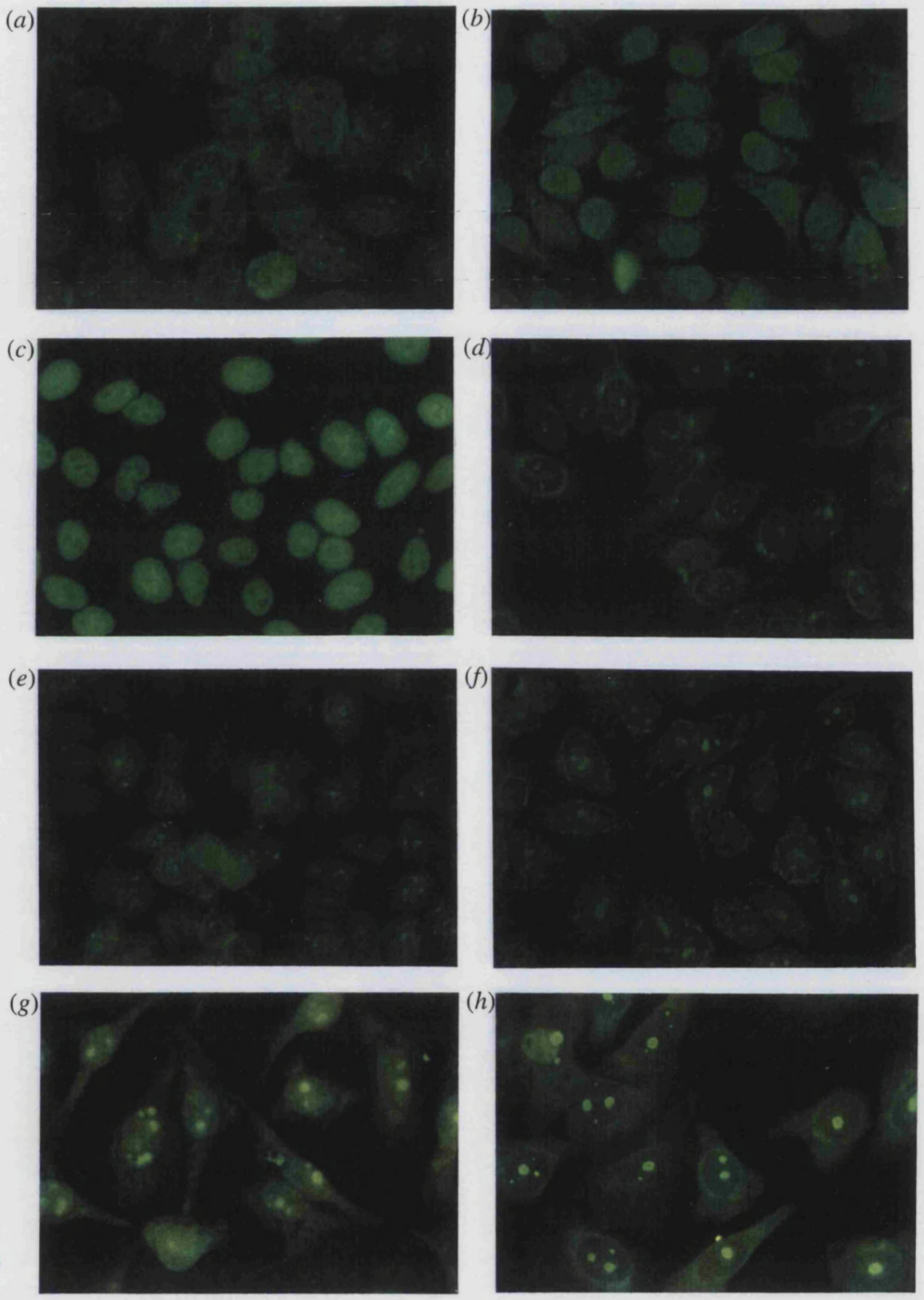
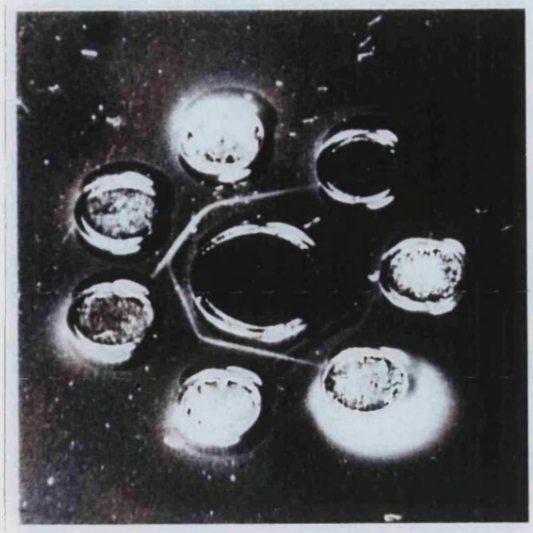
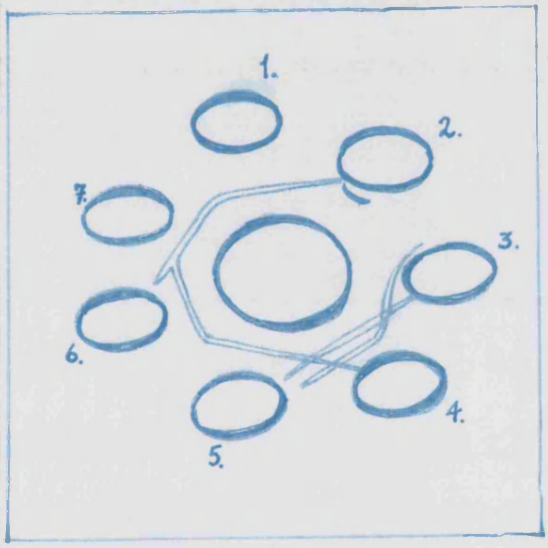


FIGURE 3.8 *(Continued)* **Indirect immunofluorescence: nuclear and nucleolar patterns produced by sera from CTD-free relatives which precipitated unidentified autoantigens by radioimmunoprecipitation.**

(*a*)



(*b*)



Key:

Central well = RTE + RLE

1. anti-Sm
2. sample LJ7
3. anti-La (wk)
4. sample LJ8
5. anti-U1 RNP
6. sample BD1
7. anti-Sm

FIGURE 3.9 Ouchterlony double immunodiffusion: identification of defined antinuclear antibody specificities in patients with connective tissue diseases. (*a*) Photograph and (*b*) schematic representation of autoantibody identification using the method of immunodiffusion. Central well, 50:50 mixture of rabbit thymus extract (RTE) and Ro/La extract (RLE); well 1, anti-Sm standard; well 3, very weak anti-La standard; well 5, anti-U1 RNP standard; well 7, anti-Sm standard. A line of identity is demonstrated between wells 1 and 7. Samples in wells 4 and 6 produced distinct precipitin lines and were counted as positive; an extremely weak precipitin line was also produced by the sample in well 2. Serum from proband BD1 (well 6) produced a line of identity with well 5 and a line of partial identity with well 7, and was judged to contain anti-U1RNP but not anti-Sm antibodies. Serum LJ8 (well 4) from the pernicious anaemia-affected father of proband LJ1 produced two precipitin lines of non-identity with well 5, and a single line of identity with well 3 is just discernible: this serum was later shown to contain anti-Ro and anti-La antibodies by both immunodiffusion and radioimmunoprecipitation (IP) techniques (Fig. 3.11, lane 9). Sample LJ7 from the healthy mother of proband LJ1 (well 2) consistently produced a very weak precipitin line, and was also found to precipitate an unidentified band by IP (Fig. 3.11, lane 10).

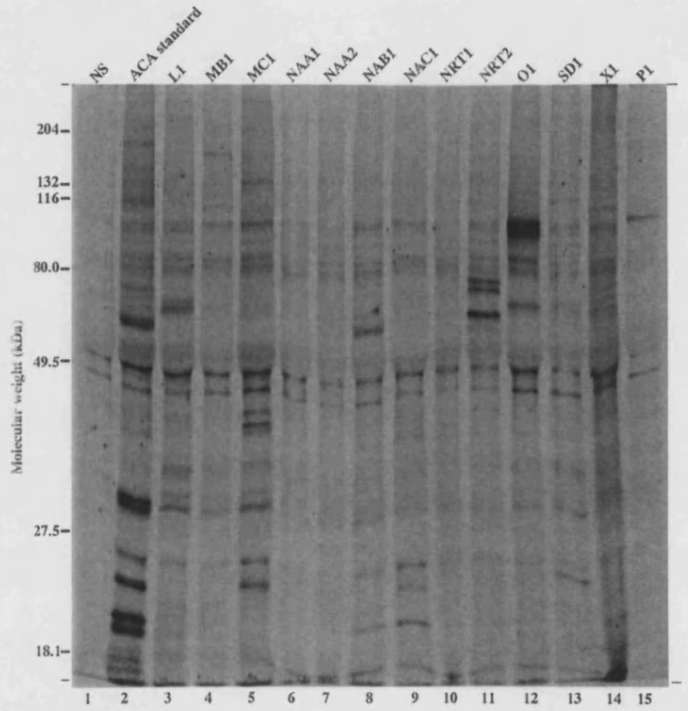


FIGURE 3.10 Radioimmunoprecipitation: sera from SSc patients which displayed the anti-centromere staining pattern by indirect immunofluorescence. Autoradiograph of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. Lane 1, normal control serum; lane 2, very high titre anti-centromere standard. Sera from SSc patients found to contain anti-centromere antibodies by indirect immunofluorescence (Table 3.1) were found to produce a variety of bands by radioimmunoprecipitation (IP) (lanes 3-15). However, the bands were generally weak, and no consistent IP profile was apparent.

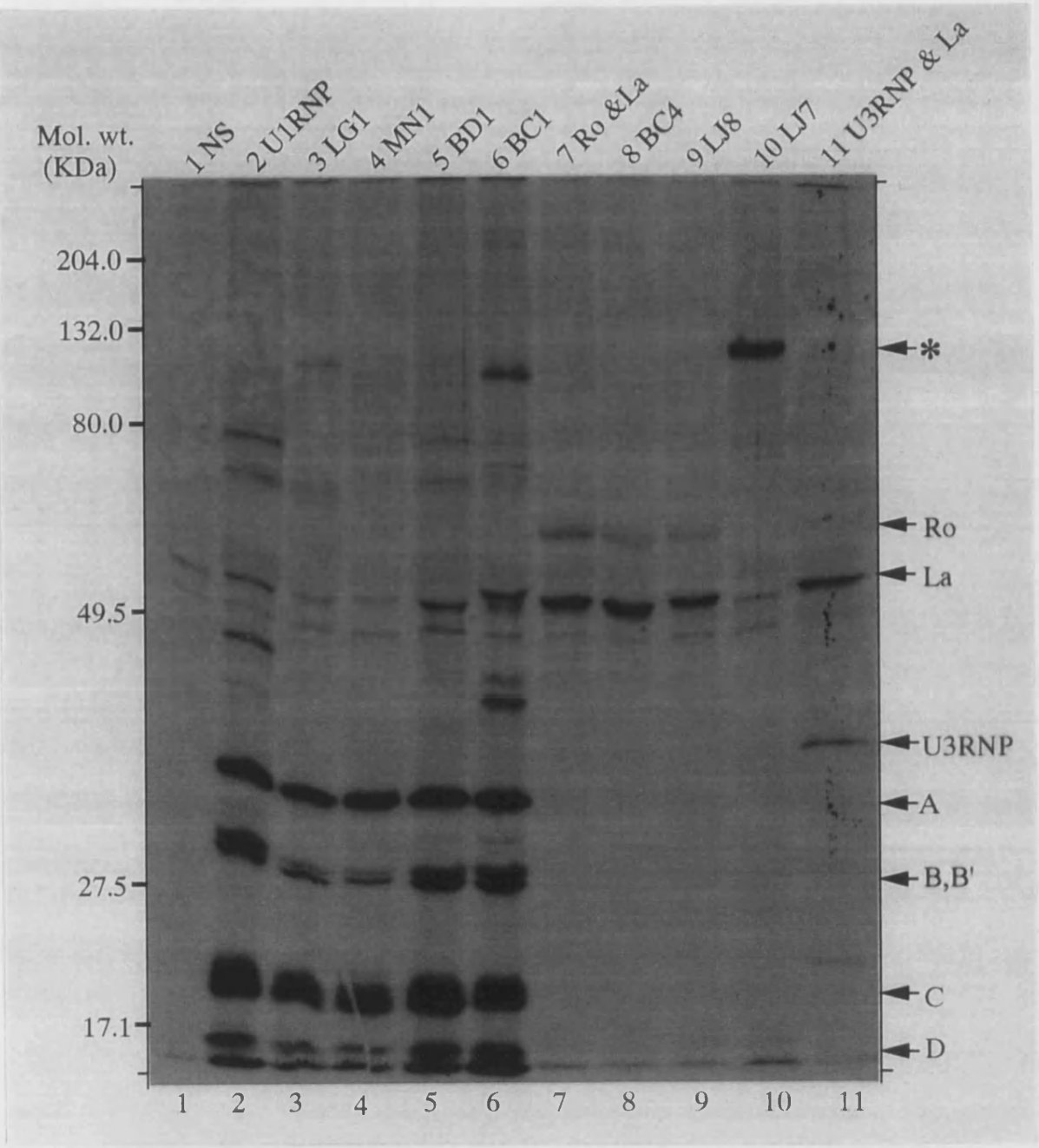


FIGURE 3.11 Radioimmunoprecipitation: confirmation of defined antinuclear antibody specificities identified by immunodiffusion in sera from SSc probands and SSc-free relatives. Autoradiograph of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. Lane 1, normal serum; lane 2, anti-U1 RNP standard; lane 7, anti-Ro/La standard; lane 11, anti-U3 RNP/La standard. Sera from SSc probands LG1, MN1, BD1 and BC1 contained antibodies to U1 RNP±Sm (lanes 3-6 respectively). Anti-Jo-1 antibodies were also identified in sample BC1 by immunodiffusion, and were confirmed by radioimmunoprecipitation (IP) (lane 6: *c.f.* Fig. 3.6, lane 4). Serum from BC4, the SLE/polymyositis-affected mother of proband BC1, contained anti-Ro and anti-La antibodies (lane 8). Serum from both the pernicious anaemia-affected father (LJ8) and the healthy mother (LJ7) of proband LJ1 both had a positive result by immunodiffusion: while anti-Ro/La antibodies were identified in serum LJ8 (confirmed here by IP (lane 9)), the specificity of serum LJ7 was not identified by either technique, although it was found to precipitate an unidentified band of ~115 kDa (*, lane 10)

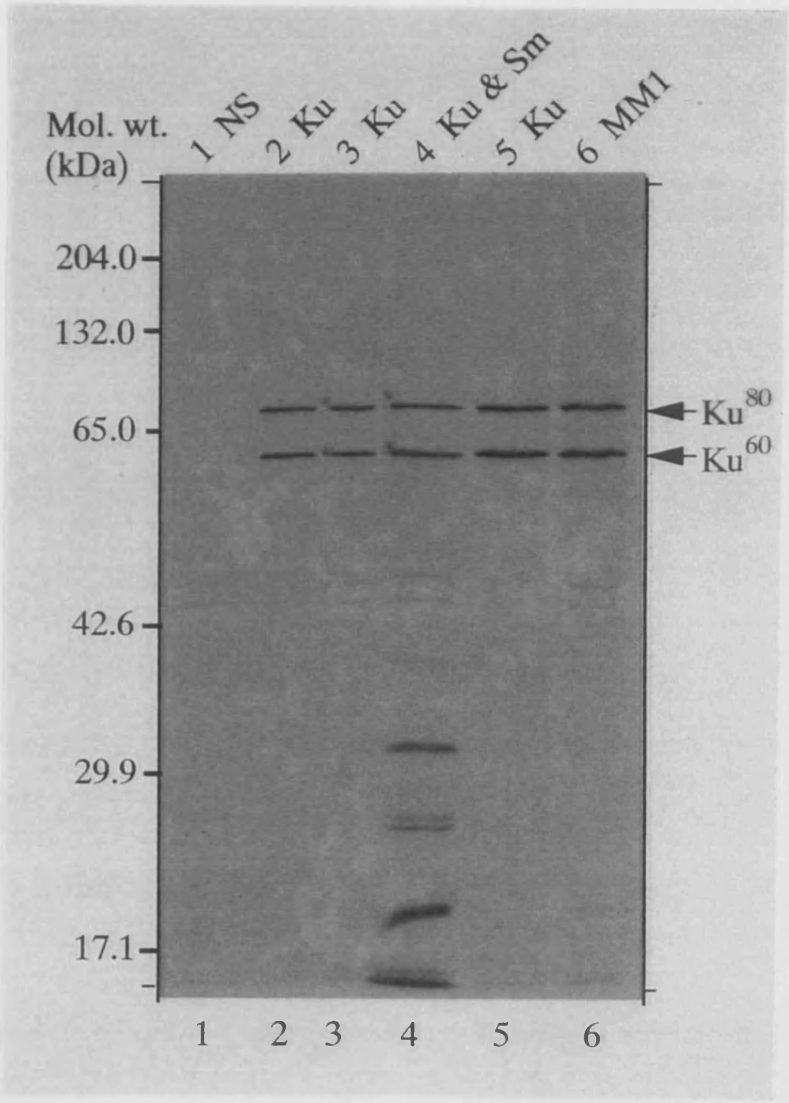


FIGURE 3.12 Radioimmunoprecipitation: identification of anti-Ku antibodies in serum from an SSc proband. Autoradiograph of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. Lane 1, normal serum; lanes 2, 3 and 5, anti-Ku standard; lane 4, anti-Ku/Sm standard. Serum from SSc patient MM1 contained anti-Ku antibodies (lane 6).

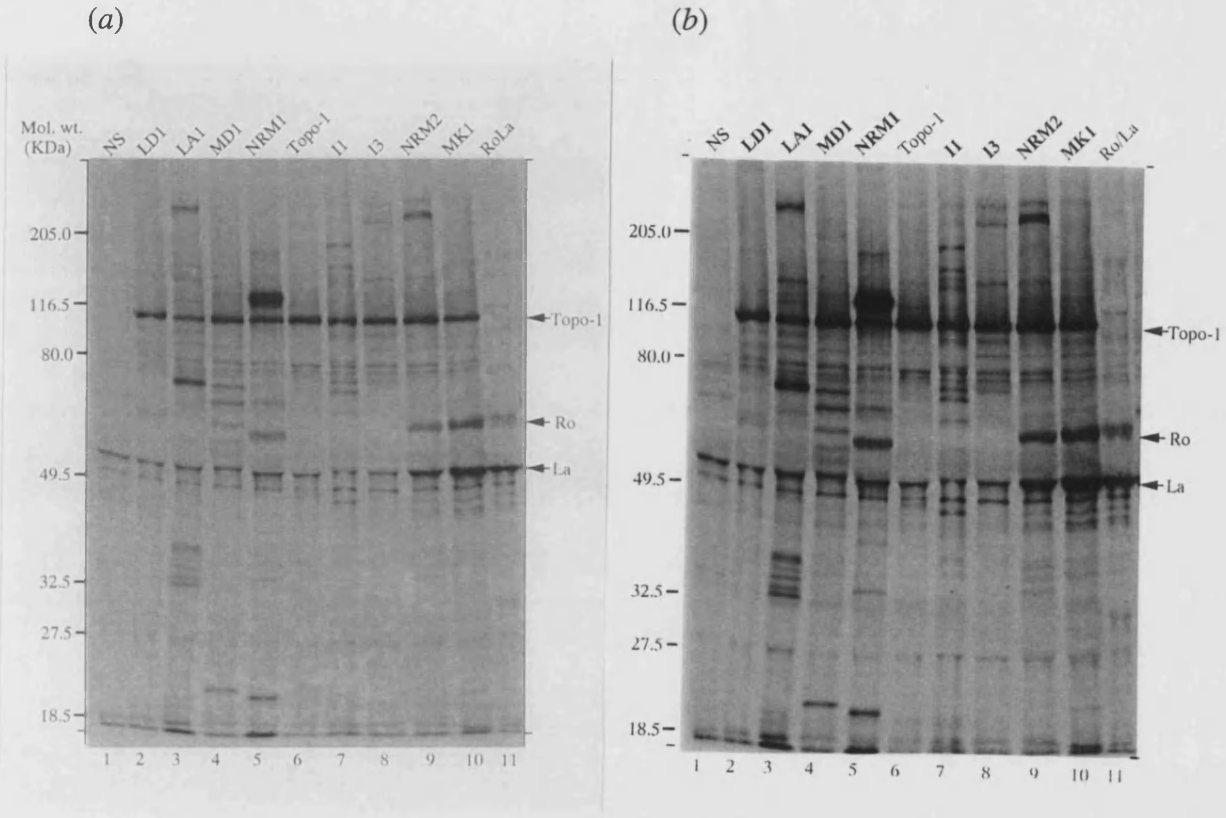


FIGURE 3.13 Radioimmunoprecipitation: identification of anti-topoisomerase I antibodies in sera from SSc patients. Autoradiograph of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. (*a*) Lane 1, normal serum; lane 6, topoisomerase I (topo I) standard; lane 11, anti-Ro/La standard. Sera from SSc patients LD1, LA1, MD1, NRM1, I1, I3, NRM2 and MK1 contained anti-topo I antibodies (lanes 2-5 and 7-10); NRM2 and MK1 sera also contained anti-Ro/La antibodies (lanes 9 and 10), while NRM1 contained anti-Ro antibodies (lane 5). In addition, serum from NRM1 precipitated strong unidentified bands of 115 and 125 kDa (a doublet was observed on autorads exposed to the same gel for shorter time periods), and further unidentified bands were precipitated by several sera, especially at higher molecular weights (*a*, lane 3 and lanes 7-9). (*b*) A longer autoradiograph exposure of the same gel showed these high molecular weight bands more clearly (*b*, lane 3 and lanes 7-9). It was hypothesized that these bands represented the large subunits of the three RNA polymerase classes.

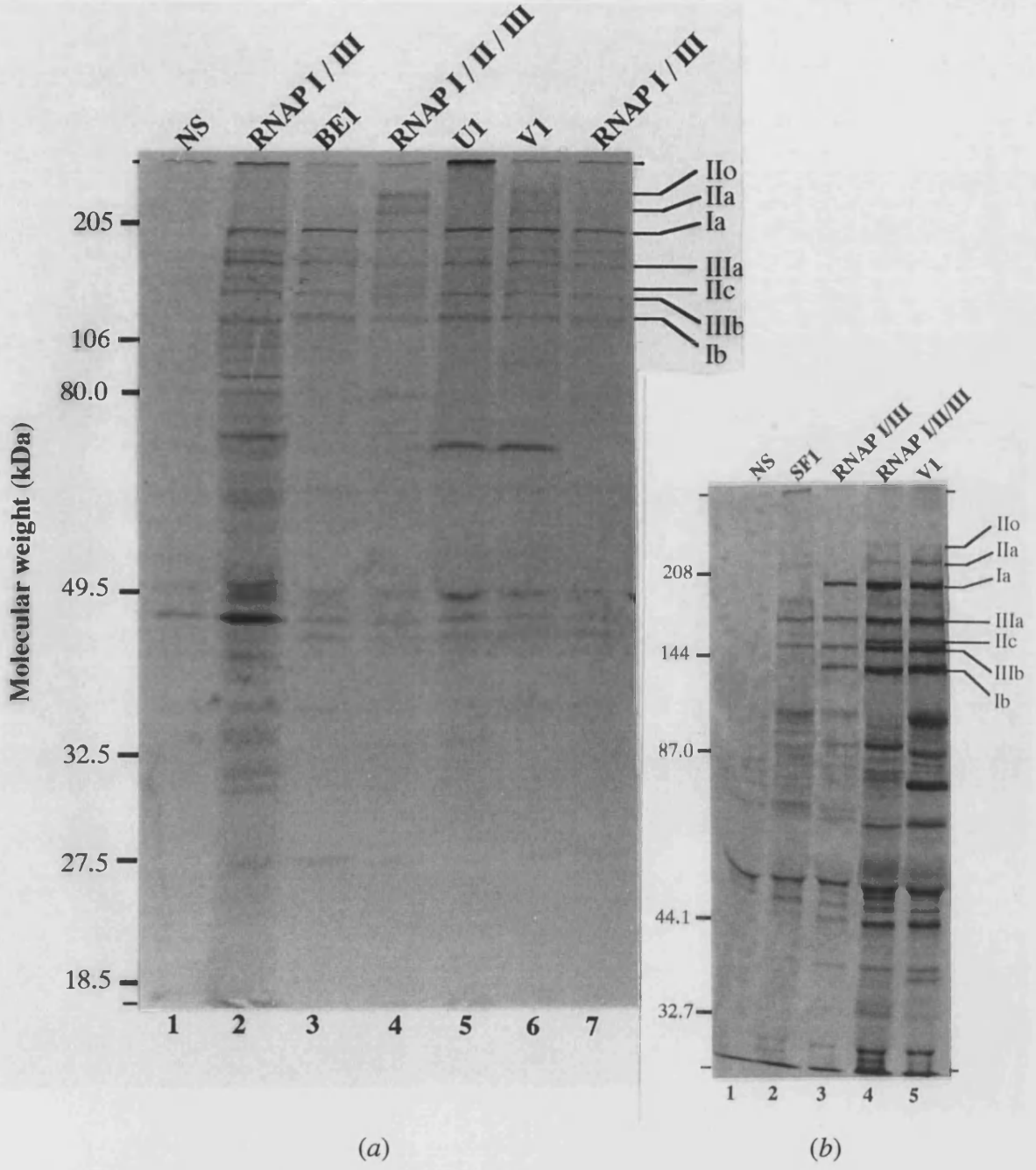


FIGURE 3.14 Radioimmunoprecipitation: identification of anti-RNA polymerase antibodies in sera from SSc patients. Autoradiograph of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. (*a*) Lane 1, normal serum; lane 2, anti-RNAP I/III standard; lane 4, anti-RNAP I/II/III standard; lane 7, anti-RNAP I/III standard. Note the bands of high molecular weight characteristic of RNAP I (subunits Ia and Ib), RNAP II (subunits IIo, IIa and IIc) and RNAP III (subunits IIIa and IIIb). Sera from SSc patients BE1 and U1 contained anti-RNAP I/III antibodies (lanes 3 and 5 respectively), while serum from patient V1 contained anti-RNAP I/II/III antibodies (lane 6). Samples were run on a 10% polyacrylamide gel. (*b*) Lane 1, normal serum; lane 3, anti-RNAP I/III standard; lane 4, RNAP I/II/III standard. Samples were run on an 8% gel. Bands precipitated by serum from SSc patient SF1 are shown in lane 2: relative mobilities are consistent with the presence of antibodies which precipitated only RNAP III, while serum from SSc patient V1 was again shown to precipitate subunits characteristic of all three RNAP enzymes.

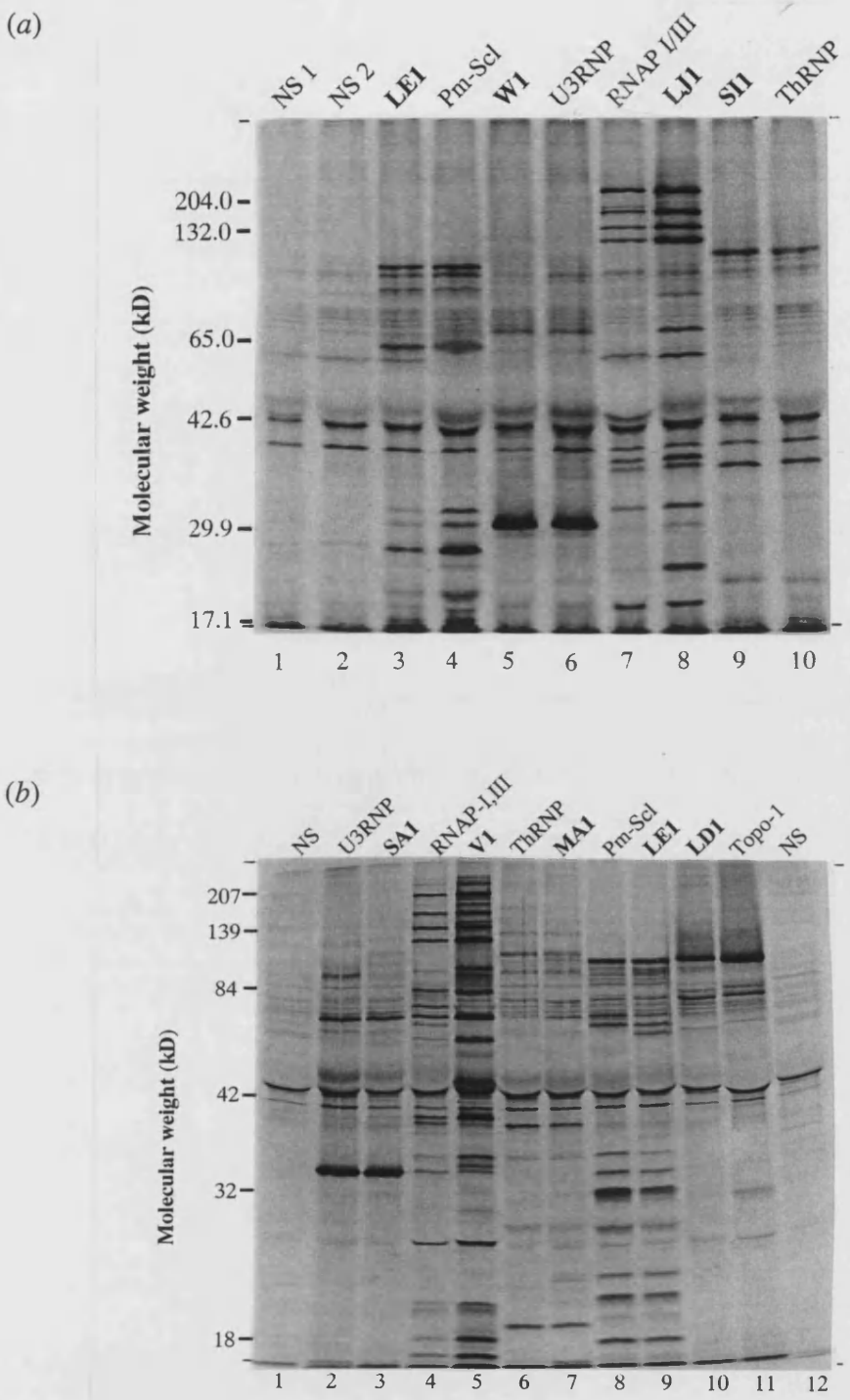


FIGURE 3.15 Radioimmunoprecipitation: identification of defined antinucleolar antibody specificities in sera from SSc patients. Autoradiograph of SDS-PAGE separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by sera attached to Protein-A Sepharose beads. (*a*) Lanes 1 and 2, normal sera; lane 4, anti-Pm-Scl standard; lane 6, anti-U3 RNP standard; lane 7, anti-RNA polymerase (RNAP) I/III standard; lane 10, anti-Th RNP standard. Sera from probands LE1, W1, LJ1 and SI1 contained antibodies which precipitated Pm-Scl (lane 3), U3RNP (lane 5), RNAPs I, II and III (lane 8), and Th RNP (lane 9), respectively. (*b*) Lanes 1 and 12, normal sera; lane 2, anti-U3 RNP standard; lane 4, anti-RNAP I /III standard; lane 6, anti-Th RNP standard; lane 8, anti-Pm-Scl standard; lane 11, anti-topoisomerase I standard. Sera from probands SA1, V1, MA1, LE1 and LD1 contained antibodies which precipitated U3 RNP (lane 3), RNAP I/II/III (lane 5), Th RNP (lane 7), Pm-Scl (lane 9) and topo I (lane 10) respectively.

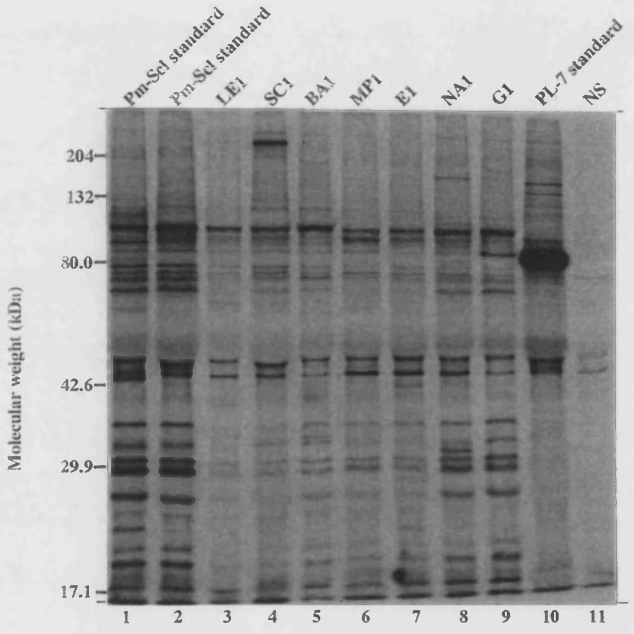


FIGURE 3.16 Radioimmunoprecipitation: identification of anti-Pm-Scl antibodies in sera from SSc patients. Autoradiograph of SDS-PAGE separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. Lanes 1 and 2, anti-Pm-Scl standards; lane 10, anti-PL-7 standard; lane 11, normal human serum. Sera from SSc probands LE1, SC1, BA1, MP1, E1, NA1 and G1 contained anti-Pm-Scl antibodies (lanes 3-9, respectively). Note the extra band of ~240 kDa precipitated by serum SC1.

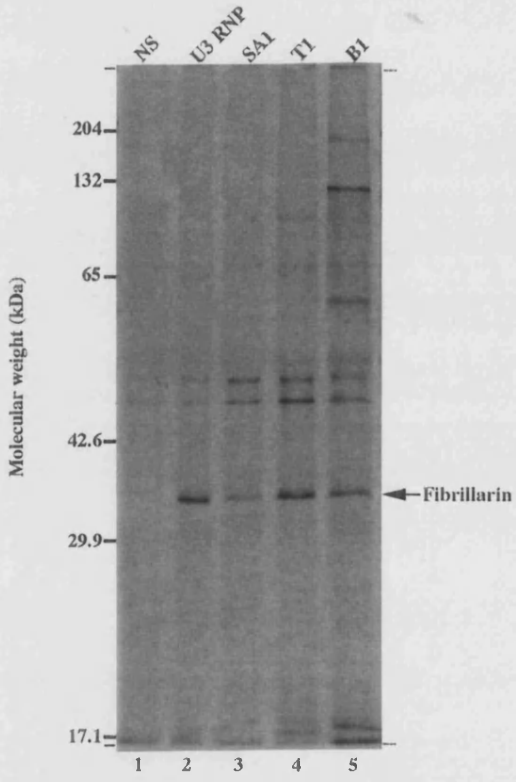


FIGURE 3.17 Radioimmunoprecipitation: identification of anti-fibrillarin (U3 RNP) antibodies in sera from SSc patients. Autoradiograph of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. Lane 1, normal human serum, lane 2 anti-U3 RNP standard. Sera from SSc probands SA1, T1 and B1 contained anti-U3 RNP antibodies (lanes 3-5, respectively). Note the extra bands precipitated by serum B1.

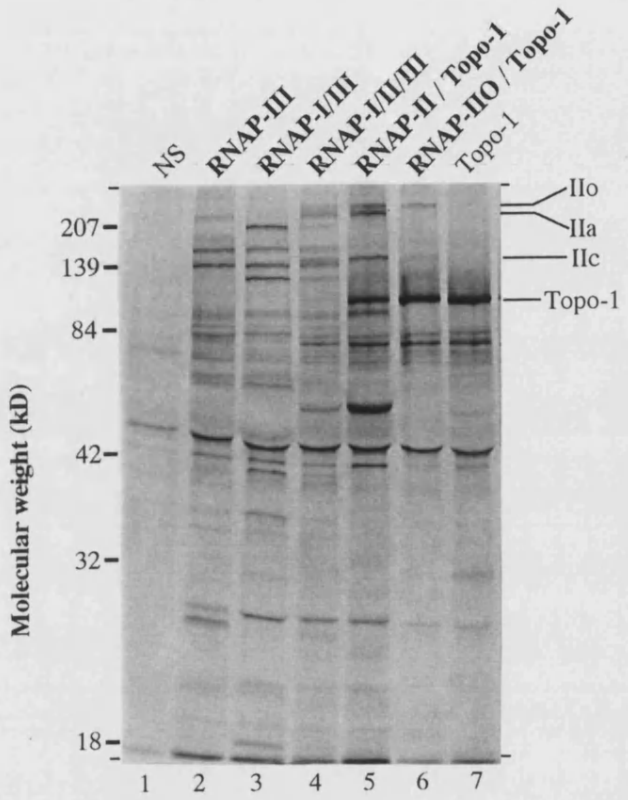


FIGURE 3.18 Radioimmunoprecipitation: co-precipitation of topoisomerase I and RNA polymerases. Autoradiograph of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. Anti-topoisomerase I (topo I) sera which precipitated bands of high molecular weight (see Fig. 3.12*b*) were included in further immunoprecipitation studies along with prototype anti-RNA polymerase (RNAP) and anti-topo I sera. Lane 1, normal serum (NS); lane 3, anti-RNAP I/III serum; lane 4, anti-RNAP I/II/III serum; lane 7, anti-topo I serum. Bands precipitated by serum from SSc patient LA1 are shown in lane 6, and are consistent with the presence of antibodies which precipitated both topo I and the phosphorylated (IIO) form of RNAP II (RNAP IIO contains subunits IIo and IIc); bands precipitated by serum NRM2 are shown in lane 5, and are consistent with the presence of antibodies which precipitated topo I, RNAP IIO and RNAP IIA (the unphosphorylated form of RNAP II, which contains subunits IIa and IIc). Meanwhile, serum from SF1, shown in lane 2, precipitated only RNAP III.

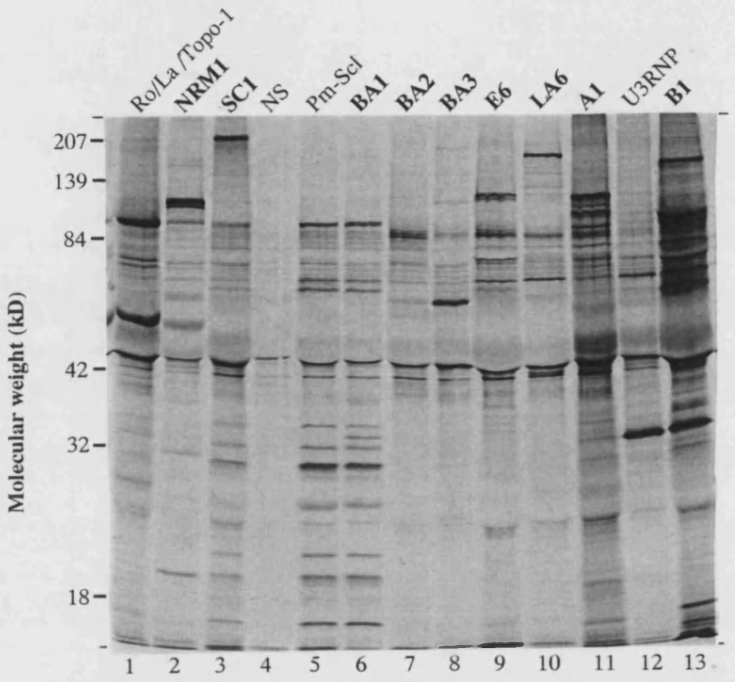


FIGURE 3.19 Radioimmunoprecipitation: sera from SSc patients and connective tissue disease-free relatives found to precipitate unidentified autoantigens. Autoradiograph of SDS-PAGE separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by sera attached to Protein-A Sepharose beads. Lane 1, anti-Ro/La/topoisomerase I standard; lane 4, normal serum; lane 5, anti-Pm-Scl standard; lane 12, anti-U3 RNP standard. Serum from proband NRM1 precipitated unidentified bands (115 and 125 kDa) and contained anti-Ro/topoisomerase I antibodies (lane 2); serum from proband SC1 precipitated an unidentified 240-kDa band and contained anti-Pm-Scl antibodies (lane 3); serum from proband BA1 contained anti-Pm-Scl antibodies (lane 6), while serum from BA2 (healthy sister of BA1) precipitated an unidentified 90-95 kDa protein triplet (lane 7), and serum from BA3 (healthy twin sister of BA1) precipitated an unidentified 65-kDa band (lane 8); serum from E6 (healthy sister of proband E1) precipitated a weak 130-kDa band (lane 9); serum from LA6 (healthy father of proband LA1) precipitated a weak 190-kDa band (lane 10); serum from proband A1 precipitated an unidentified 140-kDa band (lane 11), and serum from proband B1 precipitated several unidentified bands in addition to the fibrillarin antigen (lane 13).

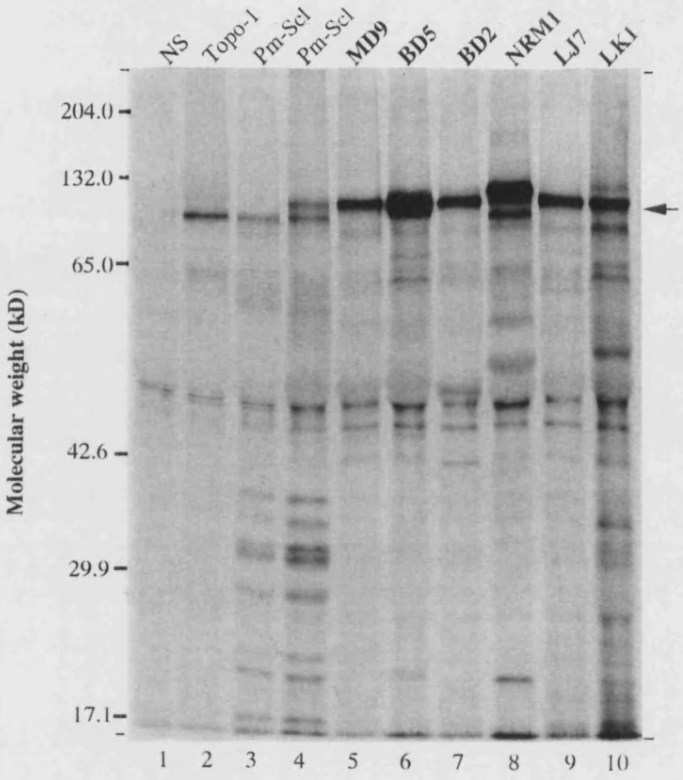


FIGURE 3.20 Radioimmunoprecipitation: sera from SSc patients and family members found to precipitate unidentified 115-kDa autoantigen/s. Autoradiograph of SDS-PAGE separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by sera attached to Protein-A Sepharose beads. Lane 1, normal serum; lane 2, anti-topoisomerase I standard (topo I); lane 3, anti-Pm-Scl standard. An unidentified 115-kDa band (arrowed) was precipitated by a prototype Pm-Scl serum from an SSc patient (lane 4). Serum from MD9 (healthy son of proband MD1), BD5 (Raynaud's phenomenon-affected sister of proband BD1), BD2 (healthy husband of BD1) and LJ7 (healthy mother of proband LJ1) also precipitated an unidentified 115-kDa band (lanes 5-7 and 9, respectively). Serum from proband NRM1 precipitated unidentified 115- and 125-kDa bands and contained anti-topo I/Ro antibodies (lane 8), while serum from proband LK1 precipitated an unidentified 115-kDa band and contained anti-Ro antibodies.

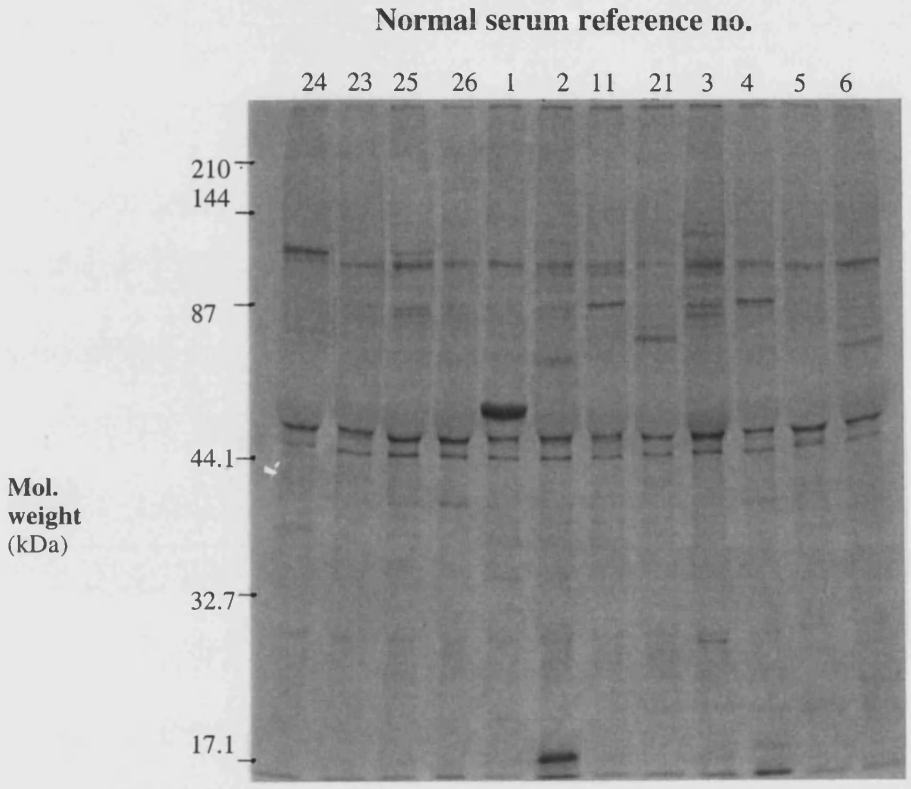


FIGURE 3.21 Radioimmunoprecipitation of sera from healthy controls. Autoradiograph of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by sera attached to Protein-A Sepharose beads. Samples NS1 and NS2 each produced a strong band, while sample NS23 produced only background bands, detected in all lanes. The remaining samples shown here produced weak or very weak unidentified bands. Samples NS1 and NS2 had both produced a '+++' nuclear staining score by IF, while sample NS23 received a '-' score for both nuclear and nucleolar staining.

3.4 DISCUSSION

Prevalence of positive immunofluorescence patterns in family members

In the original family study, carried out in 1986 (206), increased frequencies of IF-ANAs were reported in the blood-relatives of patients with SSc compared with controls (27% and 8%, respectively). The present study is one of a number of follow-up studies conducted by various members of the U.K. SSc Study Group (207,226,331,361). Although 17% of first-degree family members demonstrated IF-ANAs in the present report, this was not significantly different from the figure of 12% in controls. However, the trend towards increased IF-ANAs in first-degree relatives of SSc patients found here would be in support of previous findings by others (90,289,292,336).

It may be important that these previous studies tended to classify all IF patterns together. By contrast, in the present study, we have specifically and separately recorded IF-ANAs and IF-ANoAs. Interestingly, it was observed in the original family study that a high proportion of the IF-ANA-positive sera from family members had nucleolar staining patterns (206). In the present study, whilst the incidence of IF-ANoAs was significantly lower in the first-degree relatives of SSc patients than in the SSc patients themselves, when independently compared with a large group of age- and sex-matched normal controls, the incidence of IF-ANoAs in blood-relatives was shown to be significantly increased. In the original family study, it was apparent that sera from family members with positive ANA-IF generally had low titre IF, a finding also supported here by the fact that most of the IF-positive family members had a low IF score.

Meanwhile, despite initial indications ((206); Table 3.2), neither IF-ANAs nor IF-ANoAs were increased in the spouses of patients compared with normal controls. Together, these results would point to genetic effects being relevant to the generation of an IF-ANoA response. However, the number of spouses included in this and in the previous family study (206) was small. Furthermore, environmental effects occurring in childhood cannot be excluded.

Detection and identification of autoantibodies by immunoprecipitation

The identification of many IF-ANoAs detected in the present study was possible due to the availability of the sensitive technique of IP, which has been developed since the original family study was carried out. Radioimmunoprecipitation has proven to be a valuable method for the detection of ANoAs not identified by other techniques. With the possible exception of anti-U3 RNP antibodies (35), ANoAs of defined specificity are difficult to predict from IF tests alone. In addition, unlike ANAs, most ANoAs are not detectable by immunodiffusion techniques, or by straightforward IB.

Most sera which were subsequently found to contain antibodies recognizing the nucleolar antigens U3 RNP, Pm-Scl or Th RNP by IP techniques did display obvious nucleolar staining by IF, often with weaker staining of the nucleoplasm. However, it

seems likely that some anti-nucleolar patterns were obscured by stronger staining of the entire nucleus: in particular, all sera with anti-RNAP I antibodies also contained anti-RNAP III antibodies, and a variety of mixed nucleolar and nuclear staining patterns was therefore observed. Clearly, when testing SSc sera, the possible presence of an ANoA should be checked by IP assays wherever possible.

Disease-specific autoantibodies in SSc

The results indicated that, despite an increased incidence of IF-ANoAs in the unaffected first-degree relatives of SSc patients compared with controls, only individuals with an SSc diagnosis had a defined ANoA. Together with related studies carried out by various members of the U.K. SSc Study Group (128,226,361), the present study implies that ACAs, anti-topo I, anti-RNAP I, anti-RNAP III, anti-Pm-Scl, anti-U3 RNP and anti-Th RNP antibodies are all SSc-specific. Considering the detection of strong IF-ANoAs in some of the non-SSc sera, the fact that SSc-associated ANoAs were subsequently detected only in SSc patients seems quite remarkable. It would appear, therefore, that an SSc-associated ANoA is a reliable marker indeed of some aspect of the pathological changes characteristic of SSc. Moreover, in those families where a relative of the proband also had a CTD, any defined autoantibodies detected were consistent with their particular diagnosis (see Table 3.1). Thus, the close association of CTDs with certain autoantibodies was further confirmed.

It has been suggested previously that, in multicase SSc families, both affected individuals tend to have the same autoantibody specificity, and, further, that there is a fundamental resemblance of their disease subtype (99). Reports by the U.K. SSc Study Group concerning the present families would support this conjecture (see Table 3.1; (226,331,361)). The implication is, that the same cause, or combination of causes, (environmental or genetic) is behind the development of the disease in both of the SSc-affected members of a single family.

Mutually exclusive SSc-specific autoantibodies

Anti-nucleolar antibodies of defined specificity were mutually exclusive, as previously reported by others. Furthermore, apart from sera with antibodies to both RNAP I and RNAP III (which are known to share structural homologies), each of the SSc-specific autoantibodies (ACA, anti-topo I, anti-U3 RNP, anti-ThRNP, anti-Pm-Scl, anti-RNAP I and anti-RNAP-III) were also mutually exclusive, with the exception of a single serum.

Clinical associations of SSc-specific autoantibodies

As found by other studies, each different ANoA tended to be associated with a particular disease subtype, although the numbers of anti-U3 RNP, anti-Th RNP, and anti-Pm-Scl antibody-positive sera detected here were too small for meaningful analysis (Table 3.7). As previously reported by members of our group, in the present cohort of SSc patients, the ACA response was associated with lc-SSc (Table 3.7*a*; (226)).

Although anti-topo I antibodies were equally distributed between limited and diffuse forms of the disease, the numbers involved were small (Table 3.7*a*; (361)). The present data does, however, support an association between RNAP I antibodies and dc-SSc (Table 3.7*b*).

These results suggest that the anti-RNAP I antibody specificity, occurring in 17% of SSc patients, is the largest, and most significant, of the SSc-specific ANoAs: the remaining ANoAs (i.e. anti-Pm-Scl, anti-U3 RNP and anti-Th RNP) occurred here in only 12%, 7% and 5% of SSc patients respectively. About half of the SSc patients were found to have anti-RNAP I, anti-topo I, or ACAs. Given that, along with anti-topo I and ACAs, the anti-RNAP I response appears to define a third major subgroup of SSc patients, it was decided to concentrate on these three groups in future studies. Thus, the occurrence of anti-topo I, anti-RNAP I, and ACAs and their clinical associations were subsequently investigated in a larger group of SSc patients, and this is the subject of Chapter 4.

Co-precipitation of RNA polymerase II and topoisomerase I

Sera from four patients were found to contain antibodies which precipitated both topo I and the 240-kDa phosphorylated (IIO) form of RNAP II: Satoh *et al.* have also observed co-precipitation of topo I and RNAP II in Japanese patients with SSc (299). Since topo I is a transcription factor for RNAP II-transcribed genes (180), it appears possible, based on the present results, that anti-topo I and anti-RNAP II antibodies recognize separate epitopes that co-localize on a key multienzyme transcriptional complex (Dr N.J.McHugh, pers.comm.). The co-precipitation of topo I and Ro, seen in three of our SSc sera, was also reported by Satoh *et al.* (299). It was decided that the co-precipitation of RNAP II, topo I, and Ro also warranted further study, and this was a second aim of the work reported in Chapter 4.

Novel SSc autoantibodies

In sera from eight SSc patients, autoantibodies of a defined specificity were not demonstrated. However, the unidentified autoantigens precipitated by four of these sera (A1, K1, MO1 and NR1: Table 3.6; Fig. 3.18) may represent novel SSc-specific autoantigens occurring in a minority of SSc sera: the unusual, characteristic staining patterns produced by two of these sera are shown in Fig. 3.7*a,b*. Of the remaining four, only one was counted as negative by IF, and even this sample had discernible nuclear staining (Fig. 3.1*b*), receiving a score of '+/-'. Identification of these antigens would clearly be of interest.

Novel autoantibodies in disease-free family members

Slightly higher frequencies of both precipitating antibodies by immunodiffusion and unidentified bands on IP gels occurred in the SSc-free relatives compared with the spouses. Some CTD-free family members showed evidence of ANoAs by both

techniques. When IF and IP results were compared, three SSc-free blood-relatives (BA2, BE6 and MD9) had both strong unidentified bands by IP (Figs 3.19 and 3.20) and an antinucleolar IF score of +++ or ++++, which was greater than the nuclear IF score by at least two gradations of intensity (Fig. 3.8*f,g,h*) (Table 3.3): therefore, these unidentified autoantigens may represent nucleolar proteins. This provides further evidence that, in a minority of the first-degree relatives of SSc patients, certain genetic (and/or environmental factors) lead to the production of antibodies which recognize specific nucleolar autoantigens. Given that 40% of SSc sera produced positive nucleolar staining scores by IF, and that 41% of SSc sera were shown to recognize defined nucleolar autoantigens, it is difficult to avoid concluding that a common factor is behind the production of ANoAs by a proportion of both SSc patients and their SSc-free first-degree relatives. However, the particular nucleolar antigens recognized by these relatives appear to be different from the antigens recognized by the SSc patients themselves.

The 115-kDa bands precipitated by sera from different members of the same family (Fig. 3.20), were also of great interest: two of these sera had also been found to produce strong nuclear staining patterns by IF (Table 3.3) (Fig. 3.8*c*). The presence of an autoantibody in the spouse of an SSc patient points to environmental effects, at least in this particular family, and is especially significant in light of the previously mentioned case of apparently environmentally induced conjugal SSc (56). It was therefore decided that sera from this very interesting family would be studied in greater detail, and this was the purpose of work described in Chapter 5.

3.5 CONCLUSIONS

The nucleolus is a focal point for the immune response in SSc patients and their relatives

It has been suggested that the nucleolus may be a focal point of the autoimmune response in SSc (288,340). Such observations have been based on an analogy with SLE, where the nucleus appears to be the focus of an antigen-driven autoimmune response (125). The present results would strongly support this hypothesis, with the added point that the nucleolus may be a focus of immunity also in the blood-relatives of SSc patients. These results did not hold for SSc spouses, however. Therefore, such an effect may have a genetic basis.

As discussed earlier, the ANA response in SLE has been linked by some to the inadequate clearance of apoptotic blebs which contain nuclear antigens. In part, this effect is thought to be linked to a genetically determined complement deficiency in SLE patients. The probable presence of modified (i.e. cryptic) self-epitopes in these apoptotic blebs is likely to be an important factor leading to the breakdown of tolerance to component nuclear antigens (47). Again, by analogy, the IF-ANoA results reported

here may imply that some genetic factor is working specifically to make the nucleolus, or certain components of it, more prone to an autoimmune response in SSc patients and their blood relatives, possibly by causing the production of cryptic epitopes of nucleolar antigens. The fact that complement deficiencies have also been observed in both SSc patients and in their relatives may be of relevance here (264). Importantly, a significant association between the presence of a C4 null allele and the production of autoreactive ANAs has also been reported both in SSc patients and in their relatives (264).

However, based on the present data, it appears that the nucleolar antigens commonly recognized by SSc patients are different from those recognized by the ANoA-positive relatives. It follows that distinct sets of cryptic self-peptides have probably been presented by the patients' APCs that were not presented by the APCs of the ANoA-positive relatives. Thus, although the presence of a genetic abnormality in some first-degree relatives of SSc patients is suggested by the current data, it would appear that additional factors are present in the proband: furthermore, the likelihood is, that these additional conditions are responsible both for the development of the SSc symptoms, and for the production of disease-specific autoantibodies, as these two aspects of the disease process appear to be inextricably linked.

The SSc-specific ANoAs are markers of disease-specific pathological conditions leading to the production of the particular cryptic peptides presented on the surface of APCs

One genetic factor which is known to be associated with the development of SSc, and, more specifically, with the production of particular SSc-specific antibodies, is the presence of certain HLA alleles. In theory, if an identical pool of cryptic peptides was produced, this factor alone could explain the proposed differential display of cryptic peptides on the surface of APCs in patients versus ANoA-positive relatives. However, it has already been pointed out that the characteristic autoantibodies found in SSc are not believed to be the cause of the disease itself. Therefore, in the particular context of ANoA production, the presence of certain types of HLA molecules on the surface of APCs seems an inadequate explanation for the development of disease, and the production of disease-specific antibodies. A more satisfactory explanation is that a characteristic set of modified autoantigens has been made available to the APCs of the proband; other possibilities include the involvement of a different population of APCs in the SSc patient, or the presence of physiological factors which could have led to the use of alternative antigen processing pathways by the same type of APC (see Section 1.7). Any of these three possibilities would reflect the existence of distinct physiological or pathological conditions in the proband. This is not to say that HLA differences between different SSc patients (and between different ANoA-positive family members) do not subsequently determine the binding potential of the particular cryptic peptides available for presentation. Indeed, this effect would still explain the

association of particular SSc-specific autoantibodies with the presence of certain HLA alleles.

To summarize, the presence of one or more particular genetic or environmentally induced abnormalities in a minority of SSc-free blood-relatives appears to have lead to the production of particular sets of cryptic nucleolar epitopes, while additional abnormalities present in the SSc patients themselves have lead to the production of alternative (or additional) sets of cryptic nucleolar epitopes. These additional abnormalities are also necessary for the development of the complete disease syndrome.

So, what might these altered conditions be? Possibilities were recently reviewed by Rosen *et al.*, and include a pre-existing vascular defect, the presence of ROS from ischaemic reperfusion events, and abnormal metal-ion status. In addition, the potential role of environmental agents in the aetiology of SSc has been stressed by several groups (see Section 1.6), and the pro-inflammatory perivascular environment so characteristic of SSc may also be of relevance.

These altered states, some or all of which have converged only in the individual destined to develop SSc, may be responsible for unique nucleolar antigen fragmentation events, which, in turn, determine the particular identity of cryptic nucleolar epitopes produced and presented by APCs (288). It seems likely that one or more of these factors occurring in the absence of the others is responsible for the production of the unidentified ANoAs by the family members of SSc patients. The lack of the remaining factors being responsible for the lack of the particular SSc-associated ANoAs in the ANoA-positive family members.

Further characterization of the antinucleolar antigens recognized by disease-free first-degree relatives of SSc patients would be of great interest, as they could identify early participants in the aetiopathogenesis of the disease. Equally likely, based on the present data, is the possibility that they represent alternative responses to the same shared susceptibility factor, be it genetic or environmental; the lack of certain other of the purported pro-pathological factors mentioned above could have protected against disease onset, and may also have prevented the generation of *particular* sets of altered nucleolar antigens, with the subsequent processing and presentation events which are responsible for the generation of SSc-specific autoantibodies. The identification of the autoantigens recognized by sera from the relatives of SSc patients may help clarify these issues, and this is one aim of our future research. Meanwhile, the present study helps to confirm the status of SSc-associated ANoAs as unique markers of the specific pathological changes characteristic of SSc, as suggested by Rosen *et al.* (288).

association of particular SSc-specific autoantibodies with the presence of certain HLA alleles.

To summarize, the presence of one or more particular genetic or environmentally induced abnormalities in a minority of SSc-free blood-relatives appears to have lead to the production of particular sets of cryptic nucleolar epitopes, while additional abnormalities present in the SSc patients themselves have lead to the production of alternative (or additional) sets of cryptic nucleolar epitopes. These additional abnormalities are also necessary for the development of the complete disease syndrome.

So, what might these altered conditions be? Possibilities were recently reviewed by Rosen *et al.*, and include a pre-existing vascular defect, the presence of reactive oxygen species (ROS) from ischaemic reperfusion events, and abnormal metal-ion status. In addition, the potential role of environmental agents in the aetiology of SSc has been stressed by several groups (see Section 1.6), and the pro-inflammatory perivascular environment so characteristic of SSc may also be of relevance.

These altered states, some or all of which have converged only in the individual destined to develop SSc, may be responsible for unique nucleolar antigen fragmentation events, which, in turn, determine the particular identity of cryptic nucleolar epitopes produced and presented by APCs (288). It seems likely that one or more of these factors occurring in the absence of the others is responsible for the production of the unidentified ANoAs by the family members of SSc patients. The lack of the remaining factors being responsible for the lack of the particular SSc-associated ANoAs in the ANoA-positive family members.

Further characterization of the antinucleolar antigens recognized by disease-free first-degree relatives of SSc patients would be of great interest, as they could identify early participants in the aetiopathogenesis of the disease. Equally likely, based on the present data, is the possibility that they represent alternative responses to the same shared susceptibility factor, be it genetic or environmental; the lack of certain other of the purported pro-pathological factors mentioned above could have protected against disease onset, and may also have prevented the generation of *particular* sets of altered nucleolar antigens, with the subsequent processing and presentation events which are responsible for the generation of SSc-specific autoantibodies. The identification of the autoantigens recognized by sera from the relatives of SSc patients may help clarify these issues, and this is one aim of our future research. Meanwhile, the present study helps to confirm the status of SSc-associated ANoAs as unique markers of the specific pathological changes characteristic of SSc, as suggested by Rosen *et al.* (288).

CHAPTER 4

RESULTS, PART 2

Anti-RNA polymerase antibodies in systemic sclerosis: clinical significance, association with anti-topoisomerase I antibodies, and identification of autoreactive subunits of RNA polymerase II

4.0 SUMMARY

The occurrence of anti-topo I, anti-RNAP, and ACAs was examined in 210 SSc patients, and their clinical associations were determined. In addition, the observed occurrence of multiple ANA specificities in certain SSc sera was further investigated.

Forty-six anti-RNAP sera were detected (21.9%) and three main groups were identified: anti-RNAP I/III sera (10; 4.8%), anti-RNAP I/II/III sera (15; 7.1%), and sera precipitating the phosphorylated (IIO) form of RNAP II in the absence of RNAPs I and III (18; 8.6%). All sera in the third group also precipitated topo I, and eight of them also precipitated the unphosphorylated (IIA) form of RNAP II. A total of 44 sera were found to precipitate topo I (21.0%), and eleven sera (5.2%) precipitated both topo I and Ro (±La), five of which also precipitated RNAP IIO. A further eleven sera (5.2%) precipitated Ro in the absence of either topo I or RNAPs. Meanwhile, anti-Jo-1 and anti-U1 RNP antibody specificities (detected in eleven sera (5.2%) and 13 sera (6.2%) respectively) tended to occur alone, and sera containing ACAs (55; 26.2%) appeared to form a separate group.

Although RNAP II/topo I multienzyme complexes may occur in cell extracts, autoreactive epitopes were shown to be located on both enzymes by a combination of antigen depletion studies, and IB experiments using affinity purified antigens. Furthermore, it was demonstrated that all sera with anti-RNAP II antibodies recognized the largest RNAP II subunit in its phosphorylated form (IIo), whereas the unphosphorylated subunit (IIa) was only recognized by sera which also precipitated RNAP IIA. Therefore, at least two different sites on the largest subunit of RNAP II are recognized by SSc sera, and one of these sites is unique to the phosphorylated form.

Based on the above findings, together with analysis of the clinical associations of individual autoantibodies, and particular autoantibody profiles, it was concluded that there are three main groups of SSc patients, each characterized by the presence of a particular, mutually exclusive SSc-specific antibody (ACA, anti-topo I or anti-RNAP III), and distinguished by certain clinical features. The ACA group had significantly less diffuse disease and internal organ involvement. Anti-topo I antibodies were frequently accompanied by antibodies recognizing Ro (±La), and/or RNAP IIO (±IIA): patients in this group had an intermediate risk of dc-SSc, and a high incidence of pulmonary disease. Anti-RNAP III antibodies were usually accompanied by antibodies recognizing RNAP I, and sometimes also by anti-RNAP II antibodies: this group had the highest incidence of diffuse disease. The suggestion was, that the context in which anti-RNAP II antibodies are produced is a key aspect of their clinical significance.

Based on these data, it was further hypothesized that, in each of the three main groups of SSc patients, distinct pathological processes are occurring, which are responsible for the characteristic symptoms, for the modification of particular autoantigens and, consequently for the production of particular autoantibodies.

4.1 INTRODUCTION

The aim of the present study was to examine the occurrence of anti-topo I, anti-RNAP and ACAs in a larger cohort of SSc patients, and to determine more clearly their clinical associations. In addition, the observed co-precipitation of RNAPs, topo I, and/or the Ro antigen by certain SSc sera was further investigated.

In order to appreciate the significance of antibodies recognizing the different RNAPs, a brief description of their structure and function is required. Three different classes of eukaryotic DNA-dependent RNAP enzyme have been described, denoted I, II and III, and each is responsible for the transcription of different sets of genes (for review, (308,373)). Thus, pre-mRNA is synthesized by the nucleoplasmic enzyme RNAP II, while RNAP III, also located in the nucleoplasm, transcribes 5S rRNA and tRNA genes. The function of RNAP I is to synthesize rRNA precursors, and this enzyme is located in the nucleolus.

Eukaryotic RNAP enzymes are highly conserved (149) and have been most extensively studied in yeast. Each of the three classes of RNAP consists of two different large subunits and around ten smaller polypeptides. The large subunits of RNAPs I, II and III have approximate molecular weights of 190 and 125 kDa, 155 and 138 kDa, and 220-240 and 145 kDa, respectively (142). Meanwhile, several of the smaller subunits are identical in two or all three of the enzyme complexes (34,149,364). Although each pair of large subunits is unique, there is a some homology between classes with regard to both sequence and conformation. Thus, a single antibody may recognize either a unique or a shared RNAP epitope or subunit, and immunoprecipitate one, two or all three enzymes.

In vivo, RNAP II occurs both in a phosphorylated and an unphosphorylated form, known as RNAP IIO and RNAP IIA respectively (38). Unlike RNAPs I and III, the largest subunit of RNAP II has a characteristic carboxy terminal repeat domain (CTRD) (65), which is the site of multiple phosphorylation/dephosphorylation reactions which control intraconversion of the two forms (262,376). The largest subunit of RNAP IIO (designated IIo; *Mr* ~240 kDa) is conformationally distinct from that of RNAP IIA (subunit IIa; *Mr* ~220 kDa) (375), and the two forms of the enzyme are active at different stages of the transcription process, sometimes in a site-specific manner (192,204,262,346,356). It is therefore possible that autoantibodies could recognize either one or both types of RNAP II.

The occurrence of anti-RNAP antibodies in the sera of SSc patients has been studied by several groups (36,144,183,185,255). The consensus of these reports is that anti-RNAP III antibodies occur alone in a very small minority of sera (0-7%), and are accompanied by anti-RNAP I antibodies in a further 4-25% of patients. Meanwhile, a third group of sera is able to precipitate all three RNAPs (I, II and III) (1-5%). In these studies, other combinations of anti-RNAP antibodies were only very rarely found in individual sera (144).

Until recently, anti-topo I antibodies and anti-RNAP antibodies were considered to be mutually exclusive, and each was thought to be disease-specific (144,183,255,277). However, a more recent study by Satoh *et al.* (298,299) reported the simultaneous presence of anti-topo I antibodies and antibodies to the phosphorylated (IIO) form of RNAP II in the sera of 19.7% of Japanese SSc patients. In contrast, only 5.3% of Black SSc patients were found to co-precipitate RNAP IIO and topo I, and no anti-RNAP IIO/topo I specificities were found when 39 Caucasian sera were tested (299). In contrast, sera containing antibodies which recognized the other two RNAP classes (I and III) were not found to co-precipitate topo I. Additionally, anti-RNAP II (i.e. IIO and IIA), and anti-RNAP IIO antibodies have been detected in the sera of Japanese SLE and MCTD/overlap syndrome patients, although none of these sera contained anti-topo I antibodies (297).

Topoisomerase I is known to catalyze the relaxation of supercoiled DNA *in vivo* (354), and was recently identified as a cofactor for activator-dependent RNAP II transcription (180,230). The two enzymes may interact with each other by direct binding (180). Consequently, the presence of multi-enzyme complexes in cell extracts may account for the observed co-precipitation of topo I and RNAP II by autoantibodies in a single serum.

While results from the previous chapter would support the occurrence of anti-RNAP II antibodies in some anti-topo I-positive sera (127), this finding has not been substantiated by others: in their study of 735 SSc patients, Bunn *et al.* (36) found anti-RNAP antibodies (III, I/III or I/II/III) in 11.7% of sera by IP, and, while anti-topo I antibodies were also detectable by their IP method (36), no serum was found to precipitate both topo I and any type of RNAP enzyme.

To further investigate our own preliminary findings on this issue (126), the present study was carried out, which included a further cohort of SSc patients: using the technique of radioimmunoprecipitation, the prevalences of antibodies to the three classes of RNAP enzyme, and to topo I, were measured in the sera of 213 SSc patients. The clinical associations of the antibodies detected in this study were then examined in detail.

The presence of anti-topo I antibodies in approximately 25% of SSc sera (52,53,327,357) has been associated with diffuse cutaneous involvement (91,114,327,349,357), and with the occurrence of pulmonary interstitial fibrosis (32,52,261,327). However, most studies linking anti-topo I antibodies with dc-SSc were carried out before the characterization of anti-RNAP antibodies. Therefore, in these studies, anti-topo I-positive patients could only be considered in relation to ACA-positive patients, or, alternatively compared with anti-topo I-negative groups: the presence of ACAs has been closely linked with a particular subtype of SSc found in about 25% of patients and characterized by very limited skin involvement, often confined to sclerodactyly (20,52,53,343).

Since then, anti-RNAP I and anti-RNAP III antibodies have together been associated with an increased risk of developing dc-SSc (255,277), and with a higher incidence of renal disease (255). For the purposes of clinical comparisons, some studies have tended to classify all sera containing anti-RNAP antibodies into a single group, often due to the small numbers involved: however, similar associations with dc-SSc (36,144,183,185) and with renal involvement (36,185) have been observed. In addition, some groups have reported an association of anti-RNAP antibodies with cardiac involvement (183,185). Different clinical features in patients with anti-RNAP antibodies compared with those with anti-topo I antibodies have also been noted by some of these authors (36,185,255), including an increased incidence of diffuse disease and renal involvement in the anti-RNAP group (36,255), and a higher rate of pulmonary involvement in patients with anti-topo I (36,297). Clinical associations were also considered in the study of Satoh *et al.* (299), who found that patients with both anti-RNAP IIO and anti-topo I antibodies had a significantly higher incidence of dc-SSc than did patients who had anti-topo I antibodies alone. However, only a small number of groups have so far studied the clinical associations of ACAs, anti-topo I antibodies and anti-RNAP antibodies in the context of a single study (36,185). Based on their results, together with others' (255), one of these studies concluded that there are three main, mutually exclusive groups of SSc sera: those with ACAs, those with both anti-RNAP I and anti-RNAP III antibodies, and those with anti-topo I antibodies (36).

In the previous chapter, sera which precipitated topo I together with the Jo-1, Ro, and/or La antigens were detected in a minority of sera, some of which also contained anti-RNAP II and anti-topo I antibodies. These sera were of particular interest, since Satoh *et al.* had reported on the co-precipitation of RNAP IIO and topo I together with either U1 RNP, Ku or Ro autoantigens (299). Bunn *et al.* have also noted that these particular ANAs did not occur in any of their anti-RNAP III, anti-RNAP I/III or anti-RNAP I/II/III sera (36). Consequently, the incidence of SSc sera containing these other multiple specificities was also examined, along with their clinical associations.

4.2 MATERIALS AND METHODS

Patients and sera

A total of 210 patients fulfilling criteria for SSc were studied, and disease subtype classifications were recorded (see Section 2.1.2). Nine patients had Si-SSc (sera and clinical data kindly provided by Dr U.Haustein, University of Leipzig, Germany) (227), while the remaining cases were ostensibly idiopathic, and included the 62 patients from the family study of SSc (Chapter 3; (127,207)). Blood samples were taken from the remaining 139 patients, from which sera were prepared (Method 2.1.3), all sera being stored at -20°C before serological analysis.

For RNHRD patients, renal and lung involvements were determined according to the definitions in Section 2.1.2. Organ involvement definitions in the remaining patients have been described elsewhere (207,227).

A proportion of the RNHRD patients were available to attend Dr McHugh's CTD clinic for a more detailed clinical assessment. All members of this subgroup had idiopathic SSc. For these patients, disease subtype was also recorded according to Barnett's three-point classification system (Section 2.1.2), and organ involvement severity scores were assigned (see Appendix I).

Immunodiffusion

The 62 family study sera had already been tested by this method (Chapter 3). Ouchterlony double immunodiffusion (Method 2.3.1) was carried out on all remaining serum samples, excluding those already known to contain ACAs. Anti-centromere antibodies are not detected by this method, and sera containing ACAs are generally considered to form a mutually exclusive group (see below).

Radioimmunoprecipitation

The 62 family study sera had already been tested by IP (Chapter 3). Also, 55 (26.2%) of the sera included in this study had previously been found to contain ACAs by IF and/or IB tests (either in the family study or in previous SSc studies carried out by various members of the laboratory). Anti-centromere antibodies are not well detected by IP assays (177), and, in the family study, no ACAs had been detected in conjunction with anti-topo I, anti-RNAP antibodies or any other ANAs. Consequently, only a proportion of ACA-positive sera were tested by IP in the present study, fourteen (25% of the total) being excluded.

The remaining 134 sera were subject to IP assays using K562-cell extracts (Method 2.3.3*a*) for the detection of antibodies precipitating RNAPs, topo I, Jo-1, U1 RNP(±Sm), PL-7, Ku, Ro and/or La antigens. While several other autoantibody specificities (mainly ANoAs) were also noted, these were not the focus of this particular study, and were not confirmed or further investigated. Note that, in the minority of sera not previously tested by IF and/or IB methods, some additional ACA specificities would also be expected to occur, but these would not be detected by IP methods.

Antigen depletion of radiolabelled K562-cell extracts

Similar IP experiments were performed using aliquots of radiolabelled K562-cell extract which had been precleared of topo I. Two cycles of depletion were carried out using a prototype anti-topo I serum (Method 2.3.3*c*). For comparison, similar "depleted" and "semi-depleted" extracts were prepared using a normal human serum.

Immunoblotting of affinity purified autoantigens

Appropriate sera were then used to purify RNAP and topo I antigens for IB studies. To validate this technique, a prototype anti-U1 RNP serum was first used to purify the constituent subunits of the U1 RNP particle (Method 2.3.3*d*). Following separation by SDS-PAGE, constituent polypeptides from affinity purified U1 RNP were transferred onto a nitro-cellulose sheet. Immunoblotting was performed as described previously (Method 2.3.7) using prototype anti-U1 RNP±Sm sera of known subunit specificity: these were mainly SLE sera, but two anti-U1 RNP sera from the present study were also included. Immunoblots were later subject to autoradiography to reveal the positions of transferred polypeptides.

The same basic method was then used to prepare extracts rich in (i) RNAPs I, II and III, (ii) RNAP IIO and topo I, and (iii) RNAP III. Similar IB experiments were then carried out using sample sera and prototype sera of known autoantibody specificity.

Immunofluorescence

A representative group of sera found to contain multiple antibody specificities by IP were subject to IF (Method 2.3.2). The patterns produced were then compared with those of prototype sera containing autoantibodies of a single specificity.

4.3 RESULTS

Immunodiffusion

A total of 32 sera had anti-topo I antibodies by immunodiffusion, while antibodies recognizing Jo-1, U1 RNP±Sm, Ro and/or La antigens were detected in eight, six, eight and four sera respectively. No anti-Ku antibodies were detected by this method.

Radioimmunoprecipitation

All ANA specificities identified by immunodiffusion were confirmed on IP gels, and many other sera containing antibodies to topo I, Jo-1, U1 RNP±Sm, Ku, Ro and/or La were also identified by IP techniques (Table 4.1). Many of these samples had shown a weak positive unidentified result by immunodiffusion. A number of sera were found to precipitate more than one of these antigens. One anti-PL-7 serum was detected (see previous Fig.3.6; specificity kindly confirmed by Dr I.Targoff, Department of Medicine, Oklahoma University Sciences Centre, Oklahoma, U.S.A.).

Co-precipitation of RNA polymerases and topoisomerase I

A total of 46 anti-RNAP sera was detected by IP (Tables 4.1-4.3), representing 21.9% of SSc patients included in this study. Three main groups of sera with anti-RNAP antibodies were identified: anti-RNAP I/III sera (ten; 4.8%), sera precipitating all three RNAPs (sixteen; 7.6%), and sera precipitating the phosphorylated (IIO) form of RNAP II in the absence of RNAPs I and III (18; 8.6%) (Tables 4.2 and 4.3). All sera in the

third group also precipitated topo I, and eight of them (3.8%) also precipitated the unphosphorylated (IIA) form of RNAP II (Table 4.3). Typical examples from each group are shown in Fig. 4.1*a*, lanes 3-6. In addition, two samples precipitated RNAP III in the absence of RNAPs I and II (1.0%; Table 4.2; Fig. 4.1*a*, lane 2), and one of the anti-RNAP I/II/III sera was also found to precipitate topo I (0.5%) (as described in Chapter 3; Table 4.2; Fig. 4.1*b*, lane 8). The subunit molecular weights of unusual RNAP specificities were verified by comparison with prototype anti-RNAP and anti-topo I sera on an 8% SDS-polyacrylamide gel (Fig. 4.1*b*).

Other multiple antinuclear antibody specificities

Of the 22 sera containing anti-Ro antibodies (10.5%), eight also contained anti-La antibodies (3.8%) (Tables 4.1 and 4.4). Only one anti-La serum failed to precipitate the Ro antigen (an anti-Pm-Scl serum; Table 4.4). Of the 44 sera (21.0%) containing anti-topo I antibodies (Table 4.3), eleven (5.2%) also precipitated the Ro antigen (Fig. 4.2, lane 1), including three anti-RNAP II/topo I sera (1.4%) and two anti-RNAP IIO/topo I sera (1.0%) (Table 4.3; Fig. 4.2, lanes 3 and 4; Fig. 4.1*a*, lane 5). Notably, the Ro, La and Jo-1 antigens were only precipitated by those anti-RNAP sera which also contained anti-topo I antibodies (*c.f.* Tables 4.2 and 4.3). Despite a fairly high prevalence of sera containing anti-Jo-1 and/or anti-U1 RNP antibodies (eleven sera (5.2%) and 13 sera (6.2%) respectively; Tables 4.1 and 4.5), most were monospecific, and only one was found to co-precipitate an RNAP (one anti-RNAP IIO/topo I/Jo-1 serum) (Table 4.5). However, a small number of sera containing other multiple ANA combinations was detected (Table 4.5). Despite reports on the co-precipitation of RNAP IIO and the Ku antigen (299), only one serum (0.5%) was found to contain anti-Ku antibodies, and this was monospecific (Table 4.5).

Antigen depletion studies

A radiolabelled extract was specifically depleted of topo I by successive incubations with anti-topo I-coated Protein-A Sepharose beads (Fig. 4.3*b*, lanes 12-14), while a normal serum did not adsorb out topo I or RNAP I, II or III (lanes 1-5). All sera that contained antibodies to topo I, irrespective of whether they also contained antibodies to RNAP, were unable to precipitate topo I from the topo I-depleted extract (lanes 6-12). In contrast, sera that contained antibodies to RNAP and topo I were able to precipitate RNAP subunits from the topo I-depleted extract with the same intensity as from the undepleted extract. These findings help confirm that anti-topo I antibodies and anti-RNAP antibodies exist separately, even when found in the same serum.

Immunoblotting of affinity purified autoantigens

The technique of using affinity purified autoantigens for IB studies was validated by the preliminary experiment using prototype anti-U1 RNP±Sm sera (Fig. 4.4, lanes 2-10). All U1 RNP subunits were successfully immunoprecipitated, separated and transferred

to nitro-cellulose (Fig. 4.4*a*). The subunit specificities of all anti-U1 RNP±Sm sera tested (Fig. 4.4*b*) were consistent with results previously obtained on the same sera using whole-cell, or nuclear-enriched, K562-cell extracts (Dr Jean Whyte, pers.comm.). Results obtained with the two anti-U1 RNP sera from the present study are shown in Table 4.5, and on Fig. 4.4*b*, lanes 6 and 7.

Autoradiographs of nitro-cellulose strips containing subunits from affinity purified RNAPs I, II and III are shown in Fig. 4.5*a*. Figure 4.5*b* shows the same strips after blotting with representative anti-RNAP sera. No serum recognized any of the RNAP I or III subunits, even those sera with anti-RNAP III or anti-RNAP I/III specificities (Table 4.2; Fig. 4.5*b*, lanes 1 and 2). However, all sera with anti-RNAP II antibodies recognized the largest RNAP II subunit in its phosphorylated (IIo) form (Tables 4.2 and 4.3; Fig. 4.5, lanes 3 and 5-8), including sera which had also precipitated topo I. In addition, the unphosphorylated subunit IIa was recognized by all sera which had precipitated RNAP IIA (Tables 4.2 and 4.3; Fig. 4.5, lanes 3, 5 and 6).

Where the nitro-cellulose strips contained affinity purified topo I and RNAP IIO (Fig. 4.6*a,c*), all sera with the anti-topo I specificity recognized the 100-kDa topo I band (Table 4.3; Fig. 4.6*b*, lanes 5, 6, 9 and 10). Again, all sera which precipitated one or both forms of RNAP II recognized the IIo subunit (Tables 4.2 and 4.3; Fig. 4.6*b*, lanes 3-5, 9 and 10; Fig. 4.6*d*, lanes 3, 5, 6 and 8).

An autoradiograph of one of the nitro-cellulose strips containing subunits from affinity purified RNAP III is shown in Fig. 4.7*a*. However, no RNAP III subunits were recognized by any of the sera containing anti-RNAP III antibodies included in subsequent blotting studies (Table 4.2; Fig. 4.7*b*, lanes 2-4 and 9).

Immunofluorescence

The two sera containing only anti-RNAP III antibodies produced a characteristic fuzzy nuclear pattern (see previous Fig. 3.3*a*). Sera with either anti-RNAP I/III or anti-RNAP I/II/III antibody specificities produced a rather different pattern, with variable speckling of both nucleus and nucleolus (Fig. 4.8*a-c*, see also previous Figs 3.4*ab* and 3.5*a*). Meanwhile, anti-topo I sera produced broadly similar IF patterns, regardless of whether or not they also precipitated RNAPs: a grainy nuclear speckle with prominent nucleolar staining was produced by such sera (Fig. 4.8*d-g*). However, several of the anti-topo I/Ro ± La sera produced distinctive nucleolar staining patterns (see previous Fig. 3.5*b*).

Clinical associations of anti-RNA polymerase autoantibodies in systemic sclerosis

Tables 4.6-4.8 show the clinical details of patients whose sera precipitated (i) RNAP III, (ii) topo I, Ro and/or La antigens, and (iii) Ku, U1 RNP and/or Jo-1 antigens, respectively, and these data are summarized in Table 4.9. The remaining individuals were made up of (iv) ACA-positive patients, (v) patients whose sera contained other defined specificities (mainly ANoA-positive patients), and (vi) patients in whose serum no defined autoantibody specificities have been detected to date (Tables 4.1 and 4.9).

Clinical data from patients whose sera contained a particular individual antibody were compared with clinical data from patients in the corresponding remaining group (i.e. patients whose sera did not contain that particular antibody) (Table 4.9). Comparisons were also made between groups of sera with different antibody profiles (Table 4.9). Where appropriate, the solitary anti-RNAP I/II/III/topo I serum was excluded from these calculations, as it often fulfilled inclusion criteria for both comparison groups.

Disease subtype and cutaneous involvement

The occurrence of lc- versus dc-SSc in the different antibody groups can be compared on Table 4.9. The individual antibody which showed the most significant association with dc-SSc was anti-RNAP III (69.2%; *c.f.* remaining group, 15.2%: $P<0.001$; relative risk (RR), 4.6), and anti-RNAPs I, IIO and IIA were also each associated with the development of diffuse disease (RNAP I, 70.8%, *c.f.* remainder, 15.7%: $P<0.001$, RR, 4.5; RNAP IIO, 56.7%, *c.f.* remainder, 16.3%: $P<0.001$, RR, 3.5; RNAP IIA, 70.0%; *c.f.* remainder, 17.2%: $P<0.001$, RR, 4.1). Furthermore, patients with anti-RNAP I/II/III antibodies had the highest calculated incidence of diffuse skin involvement (84.6%; *c.f.* remaining group 18.3%, $P<0.001$; RR, 4.6). Although the anti-RNAP I/III group also showed an increased incidence of dc-SSc (50.0%), this was not significantly different from the remaining group (21.6%: $0.05<P<0.1$).

The presence of ACAs was associated with lc-SSc (93.9%; *c.f.* remaining group, 70.3%: $P<0.005$). A majority of anti-topo I sera (68.3%) also came from patients with lc-SSc, but this was not significantly different from the remaining patients (79.4%), regardless of the absence (70.8%) or presence (66.7%) of anti-RNAP II antibodies or anti-RNAP IIO antibodies (70.0%). However, when anti-topo I sera (30.0% dc-SSc excluding patient PB) were compared with the anti-RNAP I/II/III group, a highly significant difference was found ($P<0.001$). Furthermore, comparison of the anti-topo I group with the anti-ACA group also revealed a significant difference ($P<0.005$). Although the anti-topo I group was not significantly different from the anti-RNAP I/III group, upon combining all sera with anti-RNAP III antibodies (32.0% lc-SSc, excluding patient PB) and comparing with the anti-topo I-positive group, a significant difference was apparent ($P<0.02$). Together, these results indicate that the three antibody groups (i.e. sera containing anti-centromere, anti-topo I, and anti-RNAP III antibodies respectively) are significantly different from one another with regard to cutaneous manifestations.

All the remaining ANAs (anti-Ro, anti-La, anti-Jo-1 and anti-U1 RNP) had a higher than average occurrence of lc-SSc, though none was significant when compared with the respective remaining group.

When considering the associations of individual anti-RNAP antibodies with disease subtype, it was difficult to separate the influence of anti-RNAPs I and III since most anti-RNAP III sera also precipitated RNAP I. However, by comparing the anti-RNAP

I/II/III group with the anti-RNAP I/III group, the effect of the additional presence of anti-RNAP II could be assessed. Also, the separate population of anti-RNAP II/topo I sera could be compared with those sera which precipitated topo I in the absence of any RNAPs. Thus, any clinical effects associated with the additional presence of anti-RNAP II antibodies could be calculated in two different contexts. Despite the higher prevalence of dc-SSc in anti-RNAP I/II/III sera compared with the anti-RNAP I/III group (84.6% vs. 50.0% respectively), however, the difference was not significant, and anti-topo I-positive patients had a similarly low incidence of dc-SSc, regardless of the additional presence of anti-RNAP II antibodies (29.2% vs. 33.3% respectively; N.S.).

Renal involvement

The incidence of renal involvement in each of the different antibody groups is shown on Table 4.9. The individual anti-RNAP antibody groups each had a higher than average incidence of kidney involvement, but none of these results was found to be significant. Furthermore, the anti-RNAP I/II/III group (28.6% with renal involvement) also failed to show a significantly increased frequency when compared with the remaining group (10.1%). The anti-RNAP I/III group actually had a lower than average incidence of renal involvement (10.0%). When anti-RNAP-III-positive sera were considered, there was no significant difference when compared with the remaining group. Furthermore, when all anti-RNAP sera of any type were considered, and compared with the remaining group, the difference did not reach significance (20.9% vs 8.6%, respectively: $0.05<P<0.1$). However, small numbers were involved.

The highest rate of kidney involvement occurred in sera with anti-La antibodies, and this was found to be a significant association (42.9%; *c.f.* remaining group, 10.3%: $P<0.05$; RR, 4.2). Furthermore, anti-Ro antibodies were also significantly associated with renal involvement (31.2%; *c.f.* remaining group, 9.6%: $P<0.05$; RR, 3.2)

A higher than average occurrence of kidney involvement was noted for anti-topo I, anti-Jo-1- and anti-U1 RNP-positive patients, though neither was significant when compared with the respective remaining group. The relatively low incidence of kidney involvement in the ACA group (1.8%) was, however, significantly different from the remaining group (15.7%: $P<0.02$), and this was similar to previous reports (261).

The anti-RNAP I/III-positive patients and the anti-RNAP I/II/III-positive patients were not significantly different when compared with each other, and the anti-RNAP II/topo I group was not significantly different when compared with sera containing anti-topo I antibodies in the absence of anti-RNAPs. However, the anti-topo I group and the ACA group were significantly different with respect to renal involvement (19.5% vs 1.8%, respectively: $P<0.01$).

Antibody profiles with a lower than average incidence of kidney involvement included anti-Jo-1 antibodies occurring alone, anti-U1 RNP antibodies alone, anti-RNAP IIO/topo I sera, and sera which contained no identified autoantibodies: of

patients in these four groups, none had kidney involvement. However, these results were not significant when compared with the respective remaining groups.

Pulmonary involvement

The incidence of pulmonary involvement in each of the different antibody groups is also shown on Table 4.9. Pulmonary involvement was associated with anti-topo I antibodies (70.7%; *c.f.* remaining group, 44.7%: $P<0.005$; RR, 1.6), and with anti-Ro antibodies (81.2%; *c.f.* remaining group: 47.6% $P<0.025$; RR, 1.7). Furthermore, a higher incidence of pulmonary involvement occurred in those anti-topo I-positive patients whose sera also precipitated the Ro antigen (80.0%) than in anti-topo I-positive patients who did not also have anti-Ro antibodies (67.7%), although the difference between the two groups was not significant. When patients with either anti-topo I and/or anti-Ro antibodies were compared with patients whose sera contained neither antibody, an even more significant association with pulmonary involvement was found (72.3% vs. 43.0%; $P<0.001$; RR, 1.7). The ACA group had a significantly reduced incidence of pulmonary involvement (36.4%; c.f. remaining group, 56.7%: $P<0.02$), confirming previous findings (261).

The incidence of pulmonary involvement was similar in anti-topo I patients regardless of the additional presence of anti-RNAP II antibodies (75.0% vs. 66.7%: N.S.). Although the incidence of lung involvement was higher in anti-RNAP I/II/III-positive patients than in anti-RNAP I/III-positive individuals (64.3% vs. 50.0%, respectively), the difference was not significant.

The ACA group and the anti-topo I group were shown to have a significantly different incidence of lung involvement (36.4% vs. 70.7%, respectively: $P<0.001$).

None of the other comparisons which were made yielded a significant result.

TABLE 4.1 Relative frequencies of autoantibody specificities identified in the sera of SSc patients by radioimmunoprecipitation assays

Antibody specificity*	No. of patients (n=210)	
RNAP I	26	(12.4)
RNAP IIO	34	(16.2)
RNAP IIA	24	(11.4)
RNAP III	28	(13.3)
Topo I	44	(21.0)
Ro	22	(10.5)
La	9	(4.3)
Jo-1	11	(5.2)
U1 RNP±Sm	13	(6.2)
Ku	1	(0.5)
CENPs†	55	(26.2)
Other defined§	20	(9.5)
None detected¶	37	(17.6)

Figures in parentheses are percentages
*Some sera had multiple antibody specificities
†Determined by immunoblotting and/or immunofluorescence (some anti-centromere (ACA) data was provided by other members of this laboratory)
§Sera precipitating Th RNP, U3 RNP, Pm-Scl and/or PL-7
¶This group is likely to be enriched with sera containing undetected ACAs and anti-nucleolar antibodies (see text)
RNAP, RNA polymerase; topo I, topoisomerase I; CENPs, centromere proteins

TABLE 4.2 Profile of autoantibody reactivities in SSc sera found to contain anti-RNA polymerase III antibodies

Patient	Sex	Clinical details	RNAP AAbs*	Topo I AAbs*	Other defined AAbs*	Autoreactive subunits by IB¶
DG	F	dc-SSc	III	-	-	Negative
YB	F	lc-SSc	III	-	-	Negative
GM	F	lc-SSc	I, III	-	-	Negative
LC	F	lc-SSc	I, III	-	-	Negative
MY	F	dc-SSc	I, III	-	-	Negative
JHa	F	lc-SSc, Pm	I, III	-	-	ND
JBe	F	lc-SSc	I, III	-	-	ND
EF	M	lc-SSc	I, III	-	-	ND
MB	F	dc-SSc	I, III	-	-	ND
JV	F	dc-SSc	I, III	-	-	ND
EB	F	dc-SSc	I, III	-	-	ND
IL	M	dc-SSc	I, III	-	-	ND
NW	F	dc-SSc, MCTD	I, IIO, IIA, III	-	-	IIo (wk)
PBi	F	lc-SSc	I, IIO, IIA, III	-	-	IIo, IIa
ML	F	dc-SSc	I, IIO, IIA, III	-	-	IIo, IIa
JN	F	dc-SSc	I, IIO, IIA, III	-	-	IIo, IIa
VP	F	dc-SSc, OA	I, IIO, IIA, III	-	-	ND
LD	F	SSc	I, IIO, IIA, III	-	-	ND
WO	M	dc-SSc, OA	I, IIO, IIA, III	-	-	ND
MRJ	F	dc-SSc	I, IIO, IIA, III	-	-	ND
EMc	F	lc-SSc	I, IIO, IIA, III	-	-	ND
WB	F	dc-SSc	I, IIO, IIA, III	-	-	ND
JMn	F	dc-SSc	I, IIO, IIA, III	-	-	ND
JR	F	SSc-*ss*, SS, vasc.	I, IIO, IIA, III	-	-	ND
WW	F	dc-SSc	I, IIO, IIA, III	-	-	ND
MAn	F	dc-SSc	I, IIO, IIA, III	-	-	ND
MCu	F	dc-SSc	I, IIO, IIA, III	-	-	ND
PBu	M	dc-SSc	I, IIO, IIA, III	topo I†§	-	IIo, IIa (wk), topo I

*Detected by radioimmunoprecipitation
†also identified by immunodiffusion
§independently tested and confirmed positive for topoisomerase I (topo I) in functional inhibition assay by Dr J. Whyte (see text)
¶sera were tested against immunoblotting (IB) strips containing affinity purified, SDS-PAGE-separated RNAPs I, II and III, and against strips containing RNAP IIO and topo I
RNAP, RNA polymerase; AAbs, autoantibodies; dc-SSc, diffuse cutaneous SSc; lc-SSc, limited cutaneous SSc; Pm, polymyositis; MCTD, mixed connective tissue disease; OA, osteoarthritis; SSc-*ss*, SSc *sine* scleroderma; SS, Sjogren's syndrome; vasc., vasculitis; ND, not done; wk, weak reactivity

TABLE 4.3 Profile of autoantibody reactivities in SSc sera found to contain anti-topoisomerase I antibodies

Patient	Sex	Clinical details	RNAP AAbs*	Topo I AAbs*	Other defined AAbs*	Autoreactive subunits by IB¶
PBu	M	dc-SSc	I, IIO, IIA, III	topo I†§	-	IIo, IIa(wk), topo I
ZK	F	lc-SSc	IIO, IIA	topo I†§	Ro†, La	IIo, IIa, topo I
For	M	Si-lc-SSc	IIO, IIA	topo I†§	Ro†	IIo, IIa, topo I
MW	F	SSc	IIO, IIA	topo I	Ro	IIo, IIa, topo I
LE	F	dc-SSc	IIO, IIA	topo I†§	-	IIo, IIa, topo I
DS	M	lc-SSc	IIO, IIA	topo I†§	-	IIo (wk), topo I
RO	F	SSc	IIO, IIA	topo I	-	ND
DJe	M	dc-SSc, MCTD	IIO, IIA	topo I	-	ND
VS	F	lc-SSc	IIO, IIA	topo I	-	ND
IC	F	lc-SSc	IIO	topo I	Ro	IIo, topo I
AWS	F	dc-SSc, OP	IIO	topo I	Ro	IIo, topo I
JBa	F	lc-SSc, myositis	IIO	topo I†§	Jo-1†	IIo, topo I
AC	M	lc-SSc	IIO	topo I†§	-	IIo, topo I
JP	F	lc-SSc	IIO	topo I†§	-	IIo, topo I
JWi	F	lc-SSc	IIO	topo I†	-	IIo, topo I
JHw	F	dc-SSc	IIO	topo I†§	-	ND
MP	F	lc-SSc, MCTD	IIO	topo I	-	ND
CH	M	dc-SSc, myositis	IIO	topo I	-	ND
ES	F	lc-SSc	IIO	topo I	-	ND
TB	M	dc-SSc	-	topo I†§	Ro†, La†	ND
CFo	F	lc-SSc	-	topo I	Ro, La	ND
EMi	F	lc-SSc	-	topo I†	Ro, Jo-1†	ND
Ble	M	Si-dc-SSc	-	topo I†§	Ro†	ND
AG	M	lc-SSc, MCTD	-	topo I†	Ro	ND
PS	F	lc-SSc	-	topo I†§	Ro	ND
ET	F	dc-SSc, MCTD	-	topo I†	U1 RNP±Sm	ND
HC	F	lc-SSc, RA, MCTD	-	topo I†	-	topo I
JWa	F	dc-SSc	-	topo I†§	-	ND
Her	M	Si-lc-SSc	-	topo I†§	-	ND
SW	F	lc-SSc	-	topo I†§	-	ND
Koe	M	Si-lc-SSc	-	topo I†§	-	ND
Mat	M	Si-lc-SSc	-	topo I†§	-	ND
Kuc	M	Si-lc-SSc	-	topo I†§	-	ND
Sch	M	Si-dc-SSc	-	topo I†§	-	ND
Spi	M	Si-dc-SSc	-	topo I†§	-	ND
Zor	M	Si-lc-SSc	-	topo I†§	-	ND
MO	F	lc-SSc	-	topo I†	-	ND
OR	F	lc-SSc, SS, Pm/Dm	-	topo I†	-	ND
MS	M	lc-SSc	-	topo I†	-	ND
MCg	F	lc-SSc	-	topo I†	-	ND
JeC	F	dc-SSc	-	topo I†	-	ND
CFr	F	lc-SSc	-	topo I†	-	ND
RH	M	lc-SSc, RA	-	topo I	-	ND
MRm	F	SSc	-	topo I	-	ND

*Detected by radioimmunoprecipitation; †also identified by immunodiffusion
§independently tested and confirmed positive for topoisomerase I (topo I) in functional inhibition assay by Dr J. Whyte (see text)
¶sera were tested against immunoblotting (IB) strips containing affinity purified, SDS-PAGE-separated RNAPs I, II and III, and against strips containing RNAP IIO and topo I
RNAP, RNA polymerase; AAbs, autoantibodies; dc-SSc, diffuse cutaneous SSc; lc-SSc, limited cutaneous SSc; Si-SSc, silica-associated SSc; MCTD, mixed connective tissue disease; OP, osteoporosis; RA, rheumatoid arthritis; SS, Sjogren's syndrome; Pm, polymyositis; Dm, dermatomyositis; wk, weak reactivity; ND, not done

TABLE 4.4 Profile of autoantibody reactivities in SSc sera found to contain anti-Ro and/or anti-La antibodies

Patient	Sex	Clinical details	RNAP AAbs*	Topo I AAbs*	Other defined AAbs*	Autoreactive subunits by IB¶
ZK	F	lc-SSc	IIO, IIA	topo I†§	Ro†, La	IIo, IIa, topo I
For	M	Si-lc-SSc	IIO, IIA	topo I†§	Ro†	IIo, IIa, topo I
MW	F	SSc	IIO, IIA	topo I	Ro	IIo, IIa, topo I
IC	F	lc-SSc	IIO	topo I	Ro	IIo, topo I
AWS	F	dc-SSc, OP	IIO	topo I	Ro	IIo, topo I
TB	M	dc-SSc	-	topo I†§	Ro†, La†	ND
CFo	F	lc-SSc	-	topo I	Ro, La	ND
EMi	F	lc-SSc	-	topo I†	Ro, Jo-1†	ND
Ble	M	Si-dc-SSc	-	topo I†§	Ro†	ND
AG	M	lc-SSc, MCTD	-	topo I†	Ro	ND
PS	F	lc-SSc	-	topo I†§	Ro	ND
AF	F	lc-SSc, SS, RA	-	-	Ro†, La†, Pm-Scl†	ND
FS	M	lc-SSc	-	-	Ro†, La†, U3RNP	ND
DA	F	lc-SSc, SS, Pm, RA	-	-	Ro, La, Jo-1, U1 RNP±Sm	ND
MN	F	SSc	-	-	Ro, La	ND
DJo	M	SSc	-	-	Ro, La	ND
HW	F	lc-SSc, MCTD, vasc.	-	-	Ro†, U1 RNP†	ND
RS	M	lc-SSc	-	-	Ro†	ND
JL	F	SSc	-	-	Ro	ND
JHd	F	lc-SSc	-	-	Ro	ND
LN	F	SSc, SS	-	-	Ro	ND
DP	F	SSc	-	-	Ro	ND
PH	F	lc-SSc, RA, OP, vasc.	-	-	La†, Pm-Scl†	ND

*Detected by radioimmunoprecipitation
†also identified by immunodiffusion
§independently tested and confirmed positive for topoisomerase I (topo I) in functional inhibition assay by Dr J. Whyte (see text)
¶sera were tested against immunoblotting (IB) strips containing affinity purified, SDS-PAGE-separated RNAPs I, II and III, and against strips containing RNAP IIO and topo I
RNAP, RNA polymerase; AAbs, autoantibodies; lc-SSc, limited cutaneous SSc; dc-SSc, diffuse cutaneous SSc; Si-SSc, silica-associated SSc; OP, osteoporosis; MCTD, mixed connective tissue disease; SS, Sjogren's syndrome; RA, rheumatoid arthritis; Pm, polymyositis; vasc., vasculitis; ND, not done

TABLE 4.5 Profile of autoantibody reactivities in SSc sera found to contain anti-Ku, anti-U1 RNP and/or anti-Jo-1 antibodies

Patient	Sex	Clinical details	RNAP AAbs*	Topo I AAbs*	Other defined AAbs*	Autoreactive subunits by IB¶
PHod	F	lc-SSc, MCTD, RA	-	-	Jo-1†	ND
HeDa	F	dc-SSc	-	-	Jo-1†	ND
ViRi	F	lc-SSc, RA	-	-	Jo-1†	ND
VPr	F	lc-SSc	-	-	Jo-1†	ND
DaGa	F	lc-SSc, RA	-	-	Jo-1† (wk)	ND
FlLe	F	lc-SSc, Pm	-	-	Jo-1	ND
ElWh	F	SSc	-	-	Jo-1	ND
JBa	F	lc-SSc, myositis	IIO	topo I†§	Jo-1†	IIo, topo I
EMi	F	lc-SSc	-	topo I†	Ro, Jo-1†	ND
JoGo	F	lc-SSc, SLE, Pm	-	-	Jo-1†, U1 RNP†, Sm†	68K, A, B/B'#
DA	F	lc-SSc, SS, Pm, RA	-	-	Ro, La, Jo-1, U1 RNP±Sm	ND
HW	F	lc-SSc, MCTD, vasc.	-	-	Ro†, U1 RNP†	ND
JBr	F	lc-SSc	-	-	U1 RNP†	ND
HeDe	F	lc-SSc, morphea	-	-	U1 RNP†	ND
BaHe	F	lc-SSc	-	-	U1 RNP†	68K, A#
JHal	F	lc-SSc	-	-	U1 RNP†	ND
EvTu	F	dc-SSc, MCTD	-	topo I†	U1 RNP ± Sm	ND
HiHa	F	SSc	-	-	U1 RNP ± Sm	ND
MaBr	F	lc-SSc, MCTD	-	-	U1 RNP ± Sm	ND
RiCh	M	SSc	-	-	U1 RNP ± Sm	ND
ADe	F	dc-SSc	-	-	U1 RNP ± Sm	ND
KrWo	F	lc-SSc	-	-	U1 RNP ± Sm	ND
CMod	F	lc-SSc	-	-	Ku	ND

*Detected by radioimmunoprecipitation; †also identified by immunodiffusion

§independently tested and confirmed positive for topoisomerase I (topo I) in functional inhibition assay by Dr J. Whyte (see text)

¶sera were tested against immunoblotting (IB) strips containing affinity purified, SDS-PAGE-separated RNAPs I, II and III, and against strips containing RNAP IIO and topo I, or against strips containing U1 RNP subunits, as applicable

#subunits of the U1 RNP particle

RNAP, RNA polymerase; AAbs, autoantibodies; lc-SSc, limited cutaneous SSc; dc-SSc, diffuse cutaneous SSc; SLE, systemic lupus erythematosus; Pm, polymyositis; SS, Sjogren's syndrome; RA, rheumatoid arthritis; MCTD, mixed connective tissue disease; vasc., vasculitis; wk, weak reactivity; ND, not done

TABLE 4.6 Clinical details of SSc patients whose sera were found to contain anti-RNA polymerase III antibodies

	Autoantibodies detected				Organ involvement†	
Pat.	RNAPs	Topo I	Others	Disease subtype*	Kidney	Lung
DG	III	-	-	D (3)	No	No
YB	III	-	-	L (1)	Yes	No
GM	I, III	-	-	L (1)	No	No
LC	I, III	-	-	L (1)	No	No
MY	I, III	-	-	D (3)	No	Yes (1)
JHa	I, III	-	-	L (2)	No	No
JBe	I, III	-	-	L	No	No
EF	I, III	-	-	L (2)	No	Yes (2)
MB	I, III	-	-	D (3)	No	Yes (1)
JV	I, III	-	-	D (3)	No	Yes (2)
EB	I, III	-	-	D (3)	No	Yes (1)
IL	I, III	-	-	D (3)	Yes (4)	No
NW	I, IIO, IIA, III	-	-	D (3)	Yes	No
PBi	I, IIO, IIA, III	-	-	L (1)	No	No
ML	I, IIO, IIA, III	-	-	D (3)	No	Yes (2)
JN	I, IIO, IIA, III	-	-	D (3)	No	Yes (2)
VP	I, IIO, IIA, III	-	-	D (3)	Yes (1)	Yes (1)
WO	I, IIO, IIA, III	-	-	D (3)	No	Yes (2)
MRJ	I, IIO, IIA, III	-	-	D (3)	No	Yes (2)
EMc	I, IIO, IIA, III	-	-	L (2)	Yes	No
WB	I, IIO, IIA, III	-	-	D (3)	No	No
JMn	I, IIO, IIA, III	-	-	D (3)	No	Yes (1)
JR	I, IIO, IIA, III	-	-	S (0)	No	No
WW	I, IIO, IIA, III	-	-	D (3)	Yes	Yes (1)
MAn	I, IIO, IIA, III	-	-	D (3)	No	Yes (1)
MCu	I, IIO, IIA, III	-	-	D (3)	No	Yes (2)
PBu	I, IIO, IIA, III	topo I	-	D (3)	No	Yes (2)

*for details, see text

†for details of organ involvement definitions, see text; for details of organ involvement severity scoring systems, see Appendix I

Pat., Patient; RNAPs, RNA polymerases; topo I, topoisomerase I; D, diffuse cutaneous SSc; L, limited cutaneous SSc; S, SSc *sine* scleroderma

TABLE 4.7 Clinical details of SSc patients whose sera were found to contain anti-topoisomerase I, anti-Ro and/or anti-La antibodies

	Autoantibodies detected				Organ involvement†	
Pat.	RNAPs	Topo I	Others	**Disease subtype***	Kidney	Lung
PBu	I, IIO, IIA, III	topo I	-	D (3)	No	Yes (2)
ZK	IIO, IIA	topo I	Ro, La	L	No	Yes (1)
For	IIO, IIA	topo I	Ro	L	No	Yes
LE	IIO, IIA	topo I	-	D (3)	No	No
DS	IIO, IIA	topo I	-	L (2)	No	No
DJe	IIO, IIA	topo I	-	D (3)	No	Yes (1)
VS	IIO, IIA	topo I	-	L (2)	Yes	Yes
IC	IIO	topo I	Ro	L (2)	No	No
AWS	IIO	topo I	Ro	D (3)	Yes	Yes (1)
JBa	IIO	topo I	Jo-1	L (1)	No	Yes (1)
AC	IIO	topo I	-	L	No	Yes (1)
JP	IIO	topo I	-	L (2)	No	Yes
JWi	IIO	topo I	-	L (1)	No	No
JHw	IIO	topo I	-	D (3)	No	No
MP	IIO	topo I	-	L (1)	No	Yes (2)
CH	IIO	topo I	-	D (3)	Yes	Yes (2)
ES	IIO	topo I	-	L (1)	No	No
TB	-	topo I	Ro, La	D (3)	No	Yes (2)
CFo	-	topo I	Ro, La	L (1)	No	No
EMi	-	topo I	Ro, Jo-1	L (1)	Yes (1)	Yes (1)
Ble	-	topo I	Ro	D (3)	Yes	Yes
AG	-	topo I	Ro	L (1)	No	Yes (1)
PS	-	topo I	Ro	L	No	Yes (2)
ET	-	topo I	U1 RNP±Sm	D (3)	No	Yes (1)
HC	-	topo I	-	L (2)	Yes (1)	Yes (2)
JWa	-	topo I	-	D (3)	No	Yes (2)
Her	-	topo I	-	L	No	Yes
SW	-	topo I	-	L	No	No
Koe	-	topo I	-	L	No	Yes
Mat	-	topo I	-	L	No	Yes
Kuc	-	topo I	-	L	No	Yes
Sch	-	topo I	-	D (3)	No	Yes
Spi	-	topo I	-	D (3)	No	Yes
Zor	-	topo I	-	L	No	Yes
MO	-	topo I	-	L (2)	Yes (2)	No
OR	-	topo I	-	L (2)	Yes (1)	Yes (3)
MS	-	topo I	-	L	No	No
MCg	-	topo I	-	L	No	Yes (2)
JeC	-	topo I	-	D (3)	No	Yes (3)
CFr	-	topo I	-	L (1)	No	No
RH	-	topo I	-	L (1)	No	No
AF	-	-	Ro, La, Pm-Scl	L (1)	Yes (1)	Yes (2)
FS	-	-	Ro, La, U3 RNP	L (2)	No	Yes (1)
DA	-	-	Ro,La,Jo-1, U1 RNP±Sm	L (2)	Yes (1)	Yes (2)
HW	-	-	Ro, U1 RNP	L (2)	No	Yes (2)
RS	-	-	Ro	L (1)	No	No
JHd	-	-	Ro, La	L	No	Yes (2)
PH	-	-	La, Pm-Scl	L (2)	Yes (1)	Yes (2)

*for details, see text

†for details of organ involvement definitions, see text; for details of organ involvement severity scoring systems, see Appendix I

Pat., Patient; RNAPs, RNA polymerases; topo I, topoisomerase I; D, diffuse cutaneous SSc; L, limited cutaneous SSc

TABLE 4.8 Clinical details of SSc patients whose sera were found to contain anti-Ku, anti-U1 RNP and/or anti-Jo-1 antibodies

	Autoantibodies detected				Organ involvement†	
Pat.	RNAPs	Topo I	Others	Disease subtype*	Kidney	Lung
PHod	-	-	Jo-1†	L (2)	No	No
HeDa	-	-	Jo-1†	D (3)	No	Yes (++)
ViRi	-	-	Jo-1†	L (1)	No	Yes (2)
VPr	-	-	Jo-1†	L	No	Yes (++)
DaGa	-	-	Jo-1†	L (1)	No	No
FlLe	-	-	Jo-1	L (1)	No	Yes
JBa	IIO	topo I†§	Jo-1†	L (1)	No	Yes (1)
EMi	-	topo I†	Ro, Jo-1†	L (1)	Yes (1)	Yes (1)
JoGo	-	-	U1 RNP†, Sm†,Jo-1†	L (1)	No	No
DA	-	-	Ro, La, Jo-1 U1 RNP±Sm	L (2)	Yes (1)	Yes (2)
HW	-	-	Ro†,U1 RNP†	L (2)	No	Yes (2)
JBr	-	-	U1 RNP†	L	No	No
HeDe	-	-	U1 RNP†	L (2)	No	No
BaHe	-	-	U1 RNP†	L	No	Yes (++)
JHal	-	-	U1 RNP†	L	No	No
EvTu	-	topo I†	U1 RNP±Sm	D (3)	No	Yes (1)
MaBr	-	-	U1 RNP± Sm	L (1)	No	No
ADe	-	-	U1 RNP± Sm	D (3)	No	Yes (++)
KrWo	-	-	U1 RNP± Sm	L (2)	No	No
CMod	-	-	Ku	L	No	Yes (++)

*for details, see text

†for details of organ involvement definitions, see text; for details of organ involvement severity scoring systems, see Appendix I

Pat., Patient; RNAPs, RNA polymerases; topo I, topoisomerase I; D, diffuse cutaneous SSc; L, limited cutaneous SSc; NR, not recorded

TABLE 4.9 Clinical associations of defined autoantibodies detected in the sera of SSc patients

		Disease subtype†					Organ involvement§					
	n	*n*¶	Limited		Diffuse		*n*¶	Kidney		*n*¶	Lung	
Individual antibodies**												
RNAP I	26	24	7	(29.2)	17	(70.8)*5	25	5	(20.0)	25	15	(60.0)
RNAP IIO	34	30	13	(43.3)	17	(56.7)*5	31	7	(22.6)	31	20	(64.5)
RNAP IIA	24	20	6	(30.0)	14	(70.0)*5	21	5	(23.8)	21	14	(66.7)
RNAP IIIΔ	28	26	8	(30.8)	18	(69.2)*5/*8	27	6	(22.2)	27	15	(55.6)
Topo IΔ	44	41	28	(68.3)*10/*11	13	(31.7)*8/*9	41	8	(19.5)*7	41	29	(70.7)*4/*12
Ro	22	16	13	(81.2)	3	(18.8)	16	5	(31.2)*1	16	13	(81.2)*2
La	9	7	6	(85.7)	1	(14.3)	7	3	(42.9)*1	7	6	(85.7)
Jo-1	11	10	9	(90.0)	1	(10.0)	10	2	(20.0)	10	7	(70.0)
U1 RNP (± Sm)	13	11	9	(81.8)	2	(18.2)	11	1	(9.1)	11	5	(45.5)
CENPs	55	49	46	(93.9)*4/*10	3	(6.1)	55	1	(1.8)*3/*7	55	20	(36.4)*3/*12
Other defined#	21	19	15	(78.9)	4	(21.1)	19	4	(21.1)	19	13	(68.4)
Antibody profiles												
None identified	37	23	21	(91.3)	2	(8.7)	23	1	(4.3)	23	7	(30.1)
RNAP I+III	10	10	5	(50.0)	5	(50.0)	10	1	(10.0)	10	5	(50.0)
RNAP I+II+IIIΔ	15	13	2	(15.4)*11/*13	11	(84.6)*5/*6	14	4	(28.6)	14	9	(64.3)
RNAP I ± II + IIIΔ	25	23	7	(30.4)	16	(69.6)*5/*9	24	5	(20.8)	24	14	(58.3)
topo I - RNAPs	25	24	17	(70.8)*13	7	(29.2)	24	5	(20.8)	24	18	(75.0)*3
topo I+RNAPIIO- IIAΔ	10	10	7	(70.0)	3	(30.0)	10	2	(20.0)	10	6	(60.0)
topo I+RNAPIIΔ	8	6	4	(66.7)	2	(33.3)	6	1	(16.7)	6	4	(66.7)
topo I+RNAPIIO± IIAΔ	18	16	11	(68.8)	5	(31.2)*6	16	3	(18.8)	16	10	(62.5)
topo I - Ro	33	31	21	(67.7)	10	(30.0)	31	5	(16.1)	31	21	(67.7)*1
topo I + Ro	11	10	7	(70.0)	3	(30.0)	10	3	(30.0)	10	8	(80.0)
Ro - topo I	11	6	6	(100.0)	0	(0.0)	6	2	(33.3)	6	5	(83.3)
topo I and/or Ro	55	47	34	(72.3)	13	(27.7)	47	10	(21.3)	47	34	(72.3)*5
Jo-1 alone	7	6	5	(83.3)	1	(16.7)	6	0	(0.0)	6	4	(66.7)
U1 RNP (± Sm) alone	9	7	6	(85.7)	1	(14.3)	7	0	(0.0)	7	2	(28.6)
Total	210	177	136	(76.8)	41	(23.2)	182	21	(11.5)	182	92	(50.5)

Figures in parentheses are percentages

*1P<0.05; *2P<0.025; *3P<0.02; *4P<0.005; *5P<0.001 when compared with the respective remaining group

*6P<0.02 when compared with each other;*7P<0.01 when compared with each other;*8P<0.01 when compared with each other; 9P<0.01 when compared with each other; *10P<0.005 when compared with each other;*11P<0.001 when compared with each other;*12P<0.001 when compared with each other;*13P<0.001 when compared with each other (all comparisons by χ^2-test with Yates' correction where appropriate)

**Some sera had multiple antibody specificities

†for details, see text

§ for organ involvement definitions, see text

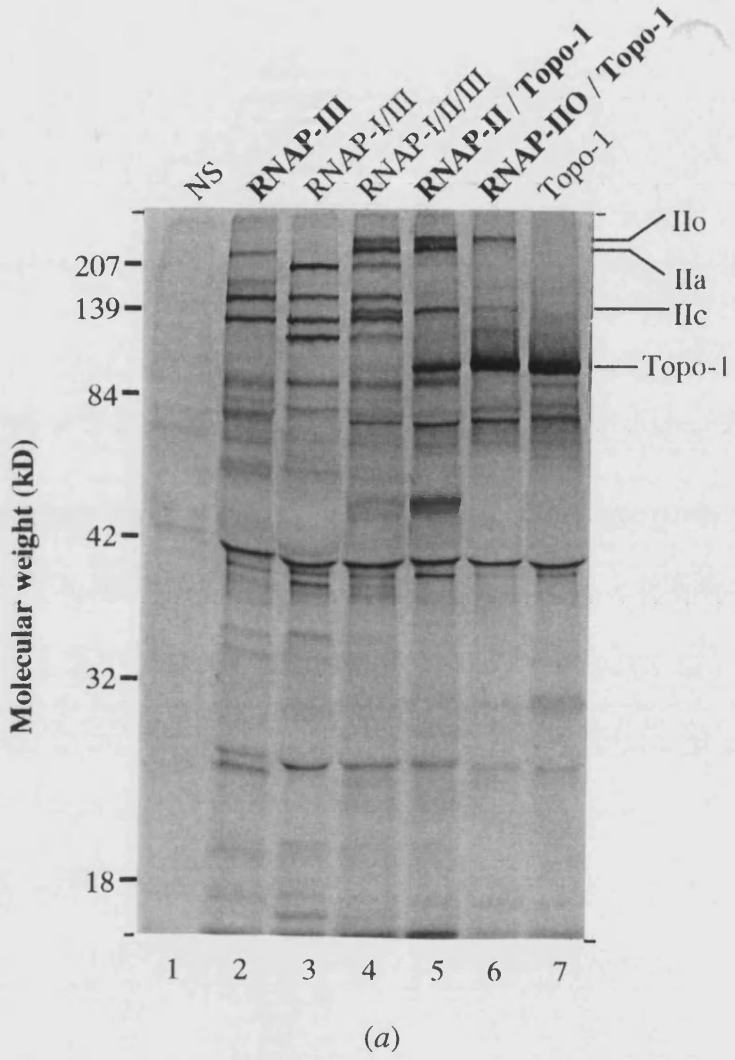
¶ total numbers of patients on whom this category of clinical information was available

Ku, Th RNP, U3 RNP, Pm-Scl and/or PL-7

Δ Where appropriate, data relating to patient PB (anti-RNAP I/II/III/topo I) were excluded from comparisons

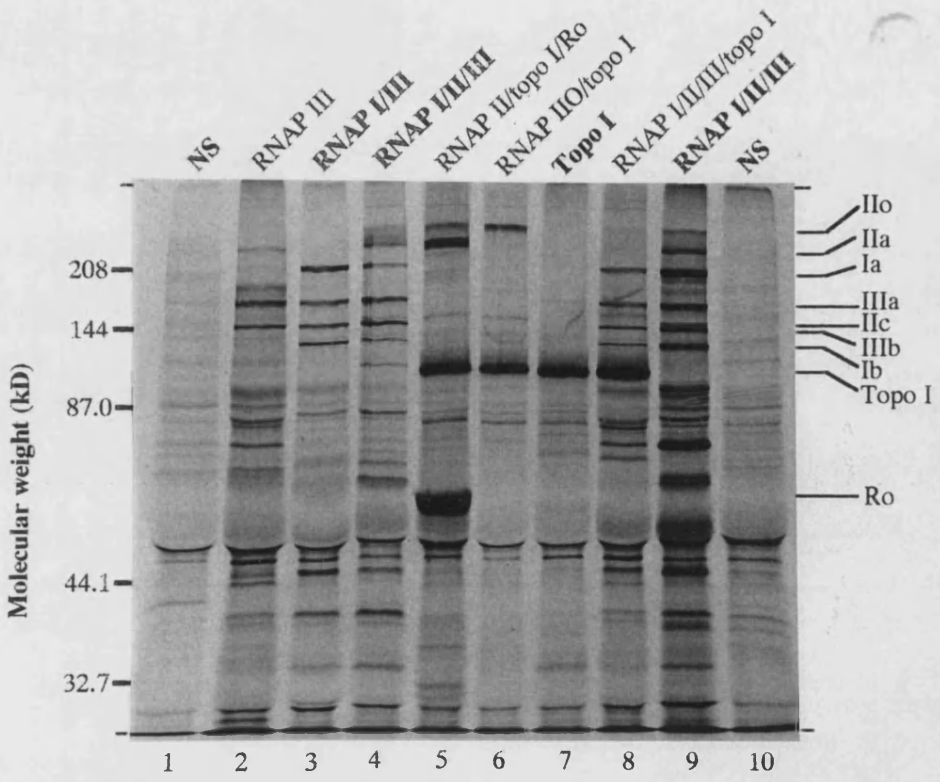
Δ Excluding patient PB (anti-RNAP I/II/III/topo I)

RNAP, RNA polymerase; Topo I, topoisomerase I; CENPs, centromere proteins



(*a*)

FIGURE 4.1 Radioimmunoprecipitation: identification of anti-RNA polymerase (RNAP) antibodies and anti-topoisomerase I (topo I) antibodies in sera from SSc patients. (*a*) Three main groups of sera were detected: sera precipitating RNAPs I and III (lane 3), sera precipitating RNAPs I, II and III (lane 4), and sera precipitating both topo I and the phosphorylated (IIO) form of RNAP II (lanes 5 and 6). Some sera in the third group also precipitated the unphosphorylated (IIA) form of RNAP II (lane 5). The characteristic large subunits of RNAP II are indicated: subunit IIc occurs in both forms of RNAP II, while the phosphorylated subunit IIo is unique to RNAP IIO, and the unphosphorylated subunit IIa is only found in RNAP IIA. A normal human serum (NS) and an anti-topo I serum are shown for comparison in lanes 1 and 7 respectively. A serum which precipitated only RNAP III is shown in lane 2 (the additional band seen at 210 kDa was later shown to be distinct from subunit IIa (see *b*)). *(Continued overleaf...)*



(*b*)

FIGURE 4.1 (*cont.*) Radioimmunoprecipitation: identification of anti-RNAP antibodies and anti-topo I antibodies in sera from SSc patients. (*b*) Samples found to have unusual RNAP specificities were then run on an 8% SDS-polyacrylamide gel to verify molecular weights. The large subunits of all three RNAPs are indicated. Two sera precipitated only RNAP III (lane 2) and one serum precipitated RNAPs I, II (weakly, but verified upon longer autoradiograph exposures), III and topo I (lane 8). The serum in lane 5 is representative of the eight sera found to precipitate RNAP II and topo I, while lane 6 represents one of the ten sera precipitating RNAP IIO and topo I. Antibodies to Ro were also detected in some of these sera (*a*, lane 5; *b*, lane 5). Comparisons were made with sera of the following specificities: normal human serum (NS, lanes 1 and 10), anti-RNAP I/III (lane 3), anti-RNAP I/II/III (lanes 4 and 9), and anti-topo I (lane 7).

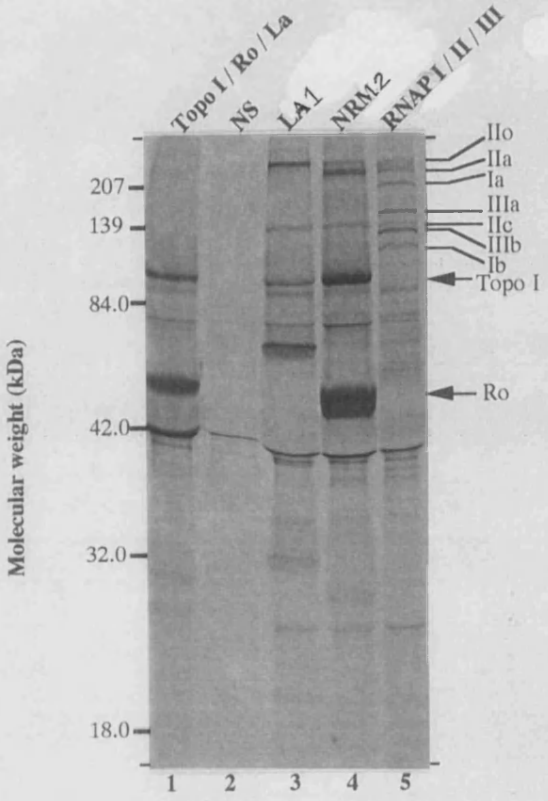
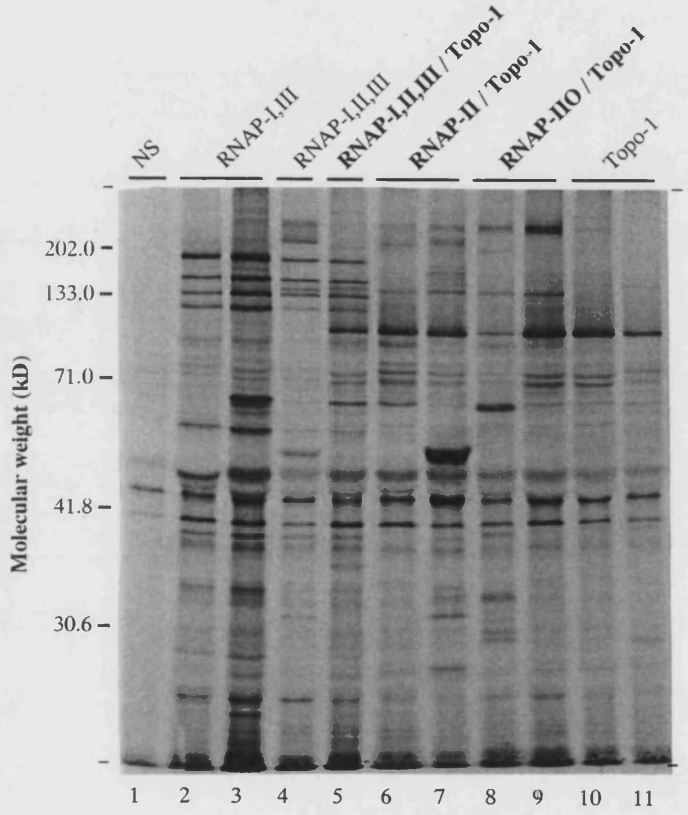
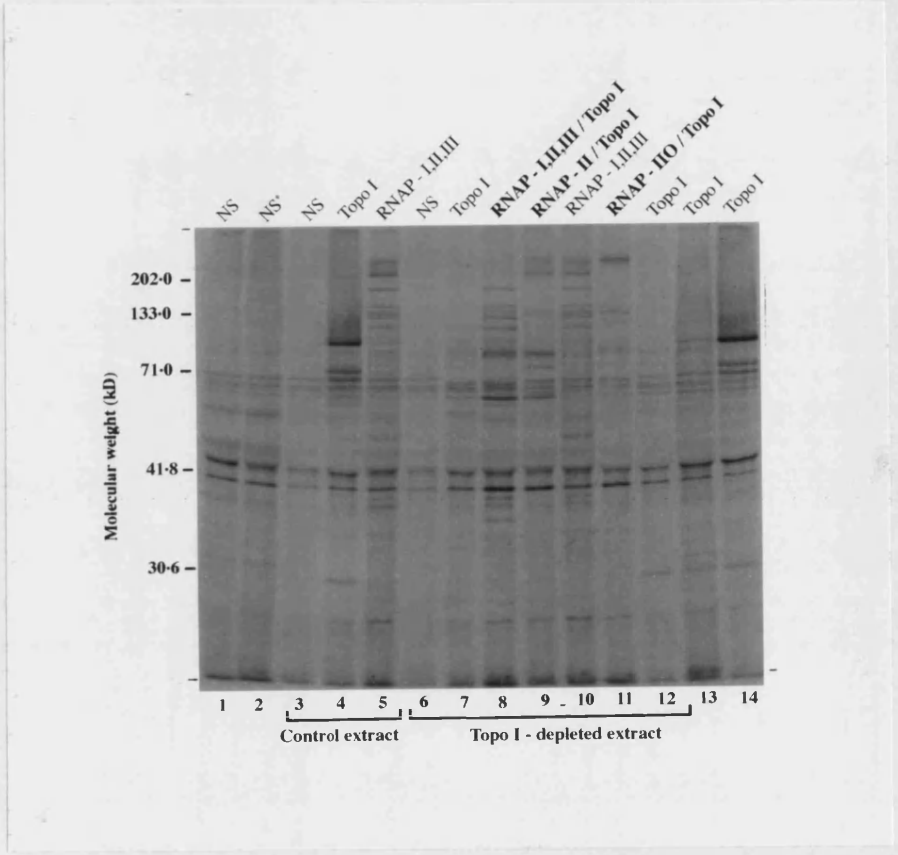


FIGURE 4.2 Radioimmunoprecipitation: Co-precipitation of RNA polymerases (RNAPs) and antinuclear antigens by sera from SSc patients. Sera precipitating RNAP III, sera precipitating RNAPs I and III, and sera precipitating RNAPs I, II and III (lane 5) did not co-precipitate other antinuclear antigens. Meanwhile, sera which precipitated RNAP IIO (± IIA) in the absence of RNAPs I and III were often found to precipitate topo I (lanes 3 and 4). Anti-topo I sera frequently also precipitated the Ro autoantigen, regardless of the additional presence or absence of anti-RNAP IIO (± IIA) antibodies (lanes 4 and 1 respectively). Lane 2, normal serum (NS).



(*a*)

FIGURE 4.3 Radioimmunoprecipitation of RNA polymerases (RNAPs) by sera from SSc patients using a K562-cell extract precleared of topoisomerase I (topo I). A batch of radiolabelled K562-cell extract was specially prepared, and divided into two lots. (*a*) Several sera previously found to precipitate topo I and/or RNAPs were then included in immunoprecipitation (IP) assays using the first lot of whole extract. *(Continued overleaf...)*



(*b*)

FIGURE 4.3 (*cont.*) Radioimmunoprecipitation of RNAPs by sera from SSc patients using a K562-cell extract precleared of topo I. (*b*) The second lot of whole-cell extract was depleted of topo I twice: the topo I band is present in the whole extract (lane 14), reduced in the semi-depleted extract (*), and absent from the topo I-depleted extract (lanes 6-12). A control sample of whole extract was "depleted" in the same way using NS (lanes 1-5), and was shown to contain both topo I (lane 4) and all three RNAPs (lane 5). Representative sera which precipitated topo I and/or RNAP from the whole extract (*a*) were then included in IP assays using the topo I-depleted fraction of extract (lanes 6-12). Topoisomerase I was no longer precipitated by sera with anti-topo I reactivity (lanes 7-9, 11 and 12). However, all RNAP subunits seen with the whole extract were still present (lanes 8-11). The same normal human serum (NS) and the anti-topo I serum are shown for comparison in lanes 6 and 12 respectively.

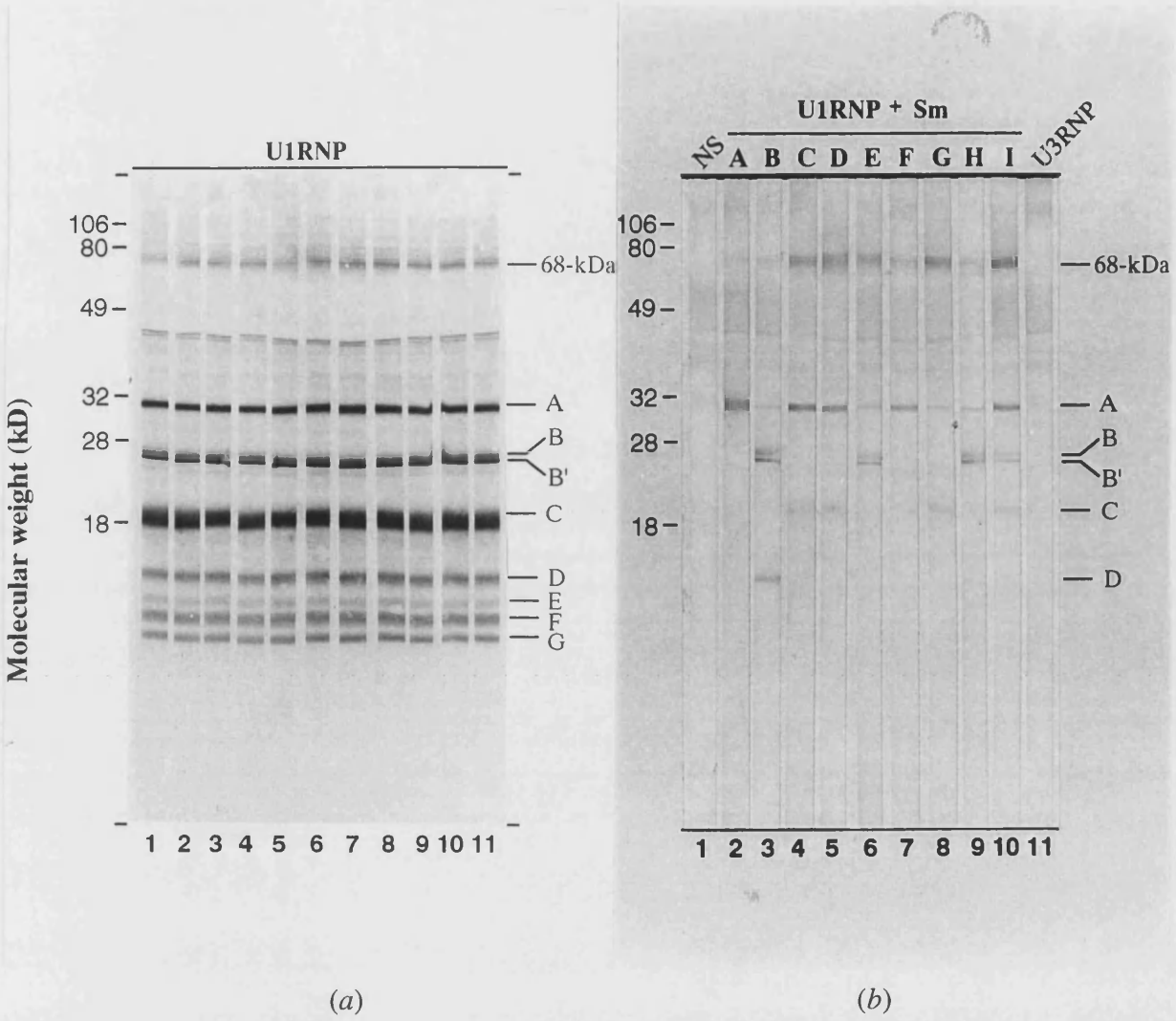
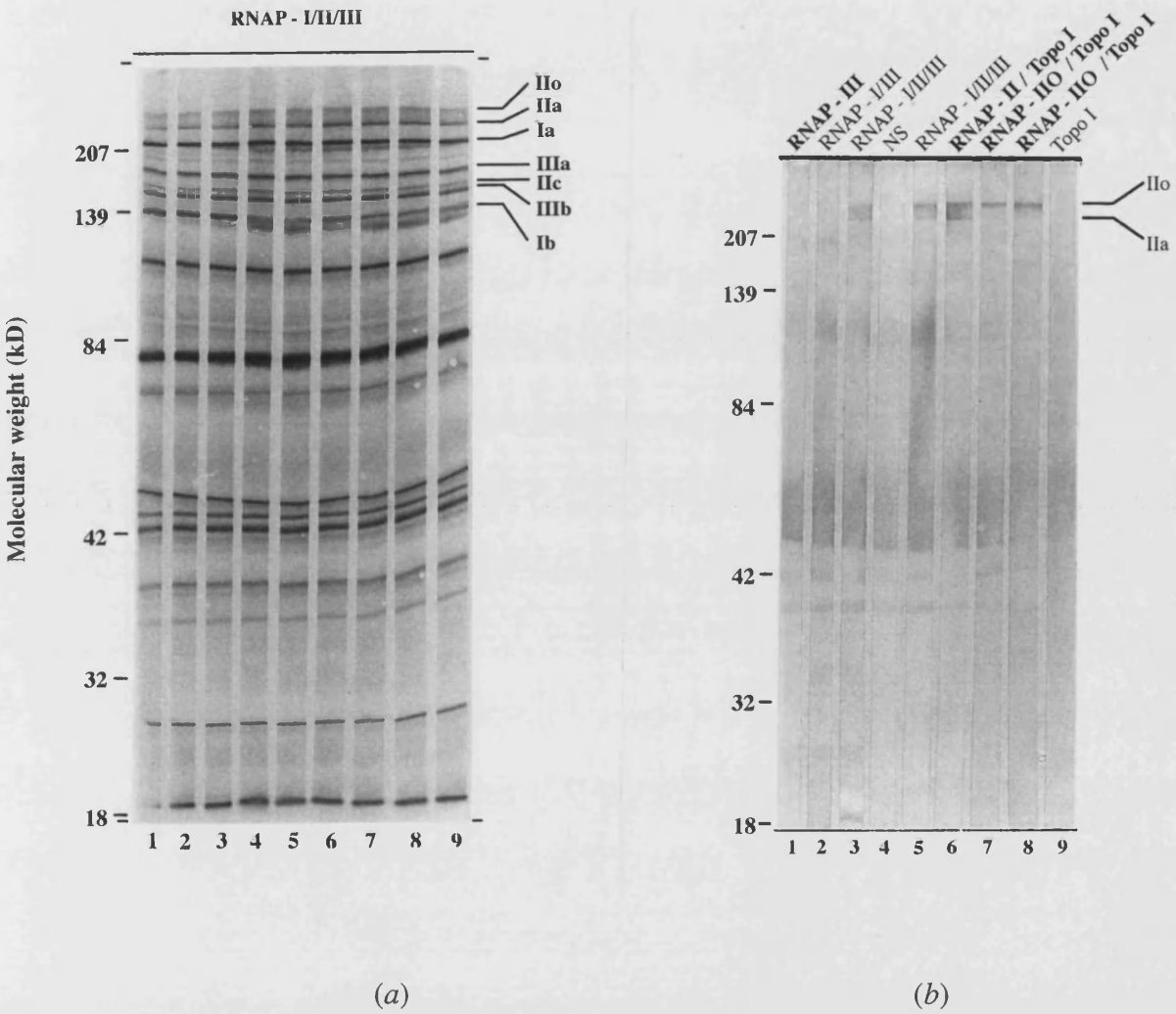
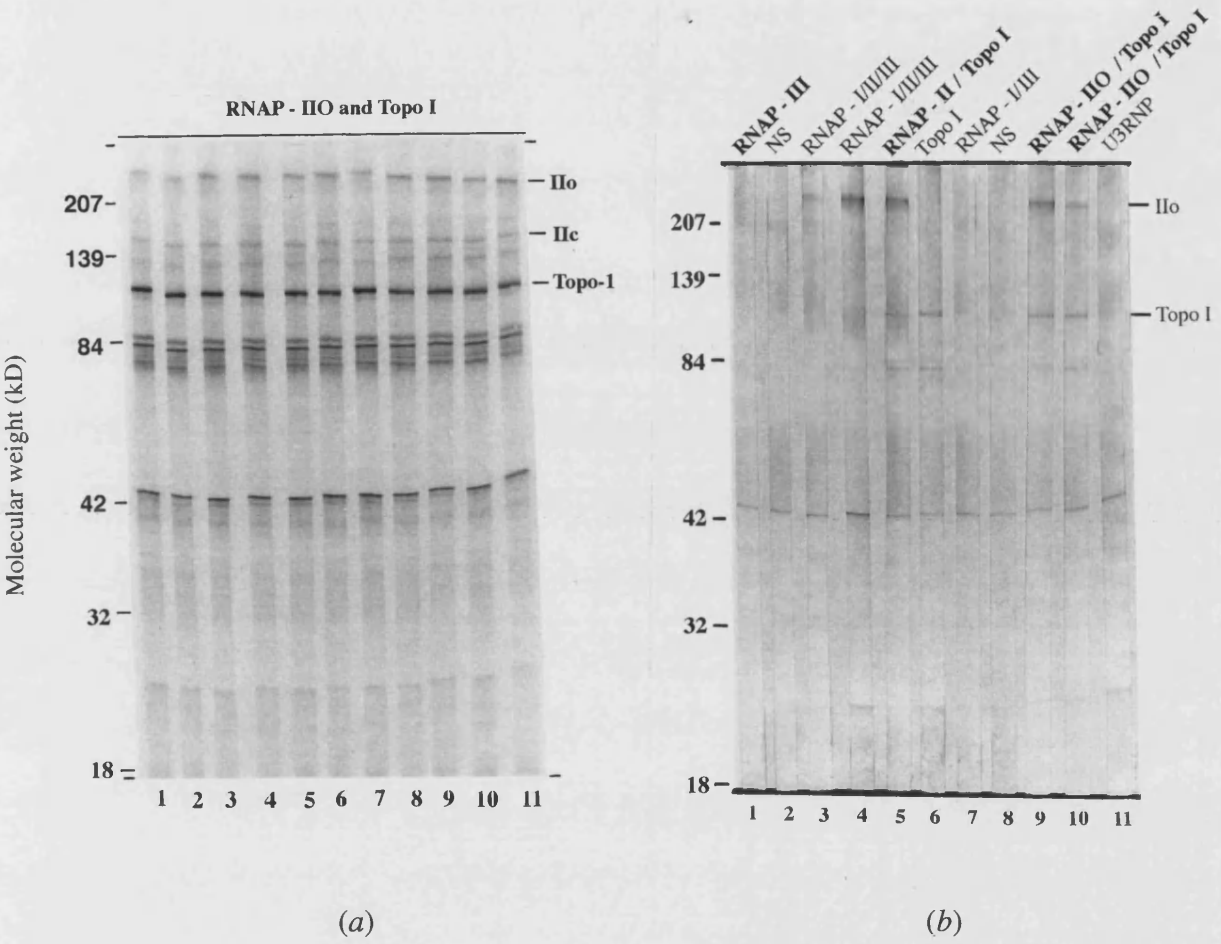


FIGURE 4.4 Immunoblotting: affinity purified radiolabelled U1 RNP subunits recognized by prototype sera containing anti-U1 RNP ±Sm antibodies. (*a*) U1 RNP was affinity purified by radioimmunoprecipitation using a prototype anti-U1RNP serum. (*b*) Following separation of constituent subunits by SDS-PAGE and their transfer to nitrocellulose, immunoblotting was carried out using prototype anti-U1 RNP±Sm sera of known specificities. Subsequent autoradiography revealed all U1 RNP subunits (A, B/B', C, D, E, F, G and the 68-kDa subunit) to be present (*a*, lanes 1-11). The subunit specificities of all prototype anti-U1 RNP±Sm sera (*b*, lanes 2, 5-7 and 8-10) were consistent with results previously obtained by immunoblotting studies using whole-cell K562 extracts. These included sera from two patients in the present study (patients JoGo and BaHe: lanes 6 and 7, respectively). The 68-kDa subunit was recognized by samples on lanes 4-10; subunit A was recognized by samples on lanes 2-10; subunits B and B' were recognized by samples in lanes 3, 6, 9 and 10; subunit C was recognized by samples in lanes 4, 5, 8 and 10, while subunit D was recognized by the sample shown in lane 3. Only background staining was observed on strips incubated with a normal human serum (*b*, lane 1: NS) and an anti-U3 RNP serum (*b*, lane 11).



(*a*) (*b*)

FIGURE 4.5 Immunoblotting: affinity purified, radiolabelled RNA polymerase (RNAP) subunits recognized by sera from SSc patients containing anti-RNAP antibodies. (*a*) The three classes of RNAP were affinity purified by radioimmunoprecipitation using a prototype anti-RNAP I/II/III serum. Subsequent autoradiography revealed all subunits of RNAPs I, II and III to be present. (*b*) Following separation of constituent subunits by SDS-PAGE and their transfer to nitrocellulose, immunoblotting was carried out using the test sera. Subsequent autoradiography of blots revealed all RNAP subunits to be present (*a*, lanes 1-9). All sera with anti-RNAP II (O and A) or anti-RNAP IIO specificities recognized the IIo subunit (*b*, lanes 3 and 5-8), while subunit IIa was recognized only by sera which had the additional anti-RNAP IIA specificity (*b*, lanes 3, 5 and 6).



(*a*) (*b*)

FIGURE 4.6 Immunoblotting: affinity purified radiolabelled topoisomerase I (topo I) and RNA polymerase (RNAP) IIO subunits recognized by sera from SSc patients containing anti-topo I and/or anti-RNAP IIO antibodies. (*a*) and (*c*) The topo I and RNAP IIO were affinity purified by radioimmunoprecipitation using a prototype anti-RNAP IIO/topo I serum. (*b*) and (*d*) Following separation of constituent polypeptides by SDS-PAGE and their transfer to nitrocellulose, immunoblotting was carried out using the test sera. The two largest subunits of RNAP-IIO and the 100-kDa topo I polypeptides are verified to be present on nitrocellulose by autoradiography (*a*, lanes 1-11; *c*, lanes 1-8). (*b*) and (*d*) All anti-topo I sera, including those with anti-RNAP specificities, recognized the 100-kDa topo I band (*b*, lanes 5, 6, 9 and 10; *d*, lanes 3, 5, 6 and 8)). Sera with anti-RNAP II or anti-RNAP IIO antibodies recognized the IIo subunit of RNAP IIO, regardless of whether they also had anti-topo I antibodies (*b*, lanes 3-5, 9 and 10; *d*, lanes 3 and 5-8). *(Continued overleaf...)*

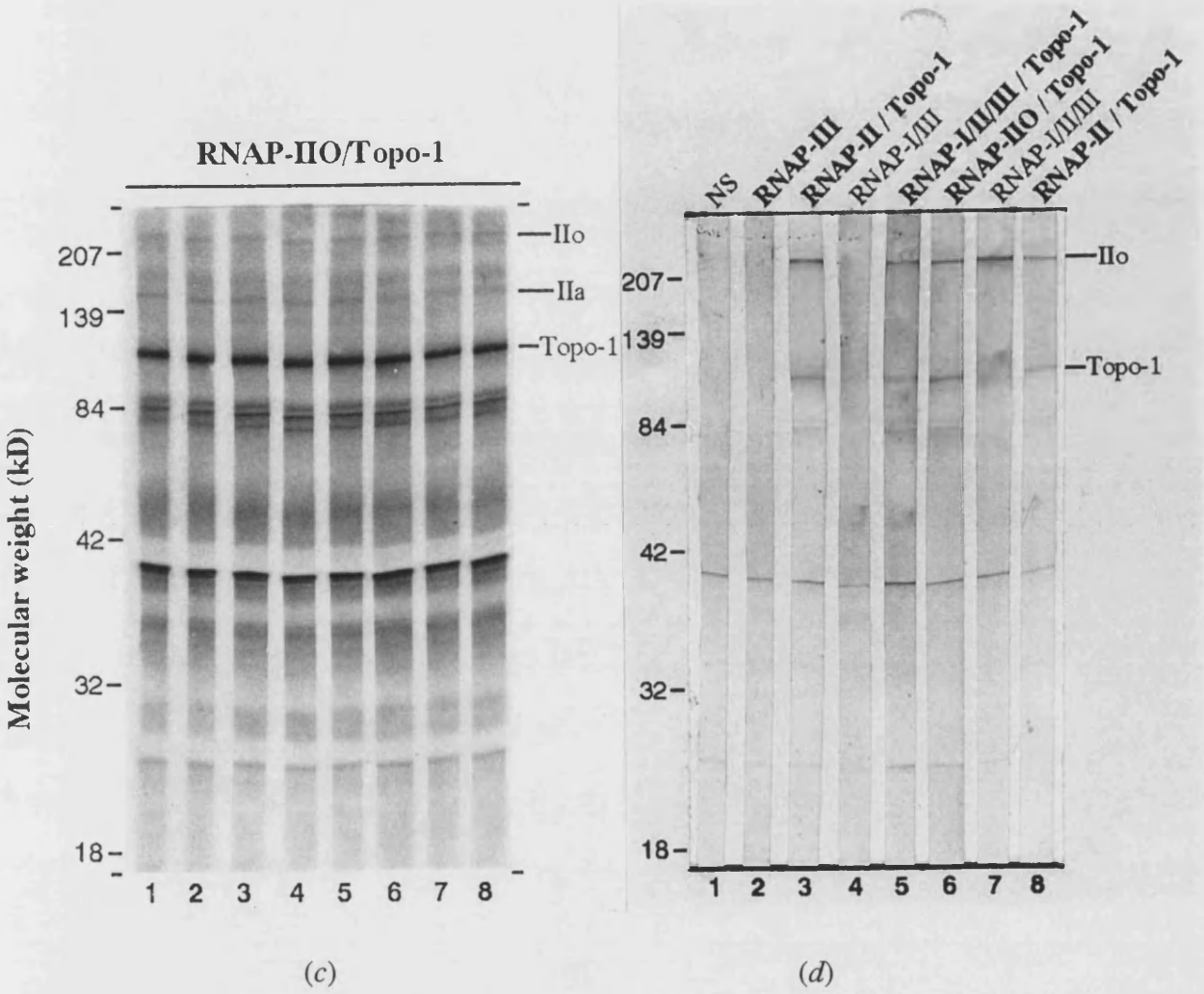
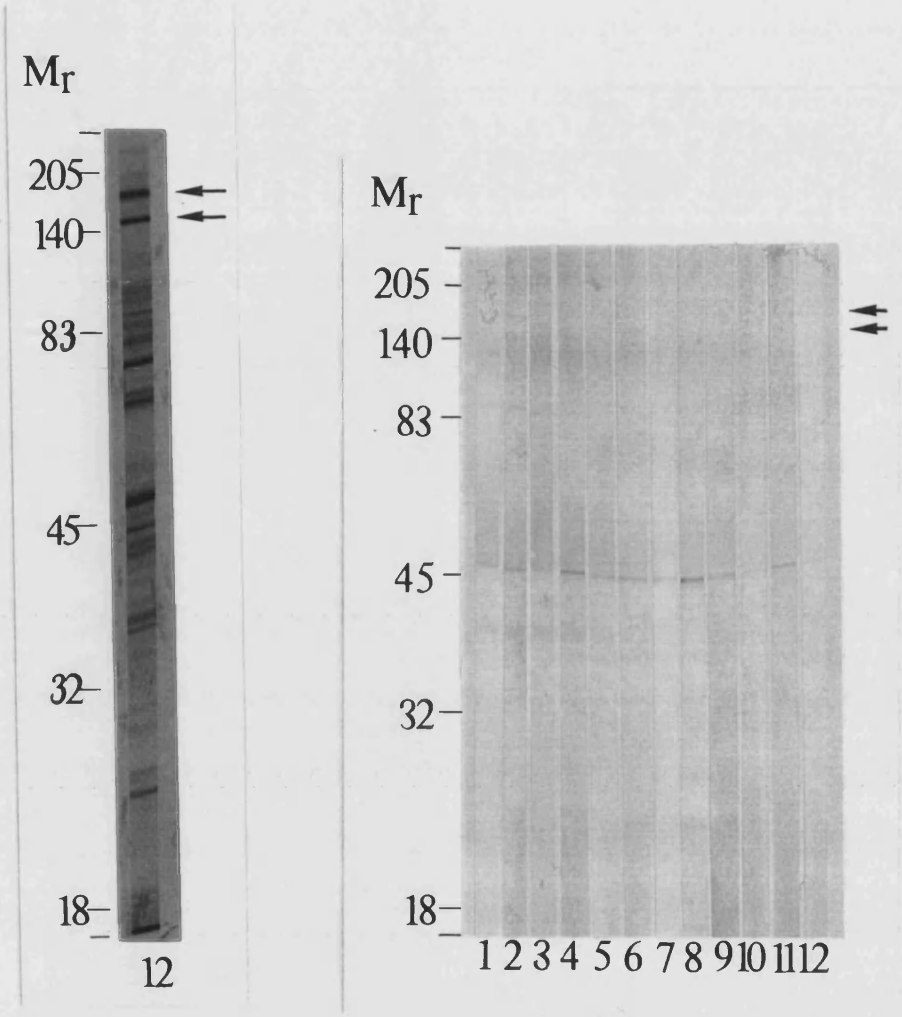


FIGURE 4.6 (*cont.*) **Immunoblotting: affinity purified radiolabelled topo I and RNAP IIO subunits recognized by sera from SSc patients containing anti-topo I and/or anti-RNAP IIO antibodies.**



Key:

1 Normal serum
2 anti-RNAP III
3 anti-RNAP I/III
4 anti-RNAP I/II/III
5 anti-RNAP II/topo I
6 anti-RNAP II/topo I
7 anti-RNAP IIO/topo I
8 anti-RNAP IIO/topo I
9 anti-RNAP I/II/III/topo I
10 anti-topo I
11 anti-U3 RNP

FIGURE 4.7 Immunoblotting: affinity purified radiolabelled RNA polymerase (RNAP) III subunits recognized by sera from SSc patients containing anti-RNAP III antibodies. (*a*) RNAP III was affinity purified by radioimmunoprecipitation using an anti-RNAP III serum. (*b*) Following separation of constituent subunits by SDS-PAGE and their transfer to nitrocellulose, immunoblotting was carried out using the test sera. Subsequent autoradiography of blots revealed all RNAP III subunits to be present (*a*, lanes 1-11). However, no serum with anti-RNAP III antibodies recognized any of the RNAP III subunits (*b*, lanes 2-4 and 9). Sera containing antibodies to RNAP IIO, RNAP IIA and/or topo I also gave a negative result (lanes 5-8). A normal serum and an anti-U3 RNP serum are included for comparison in lanes 1 and 11 respectively.

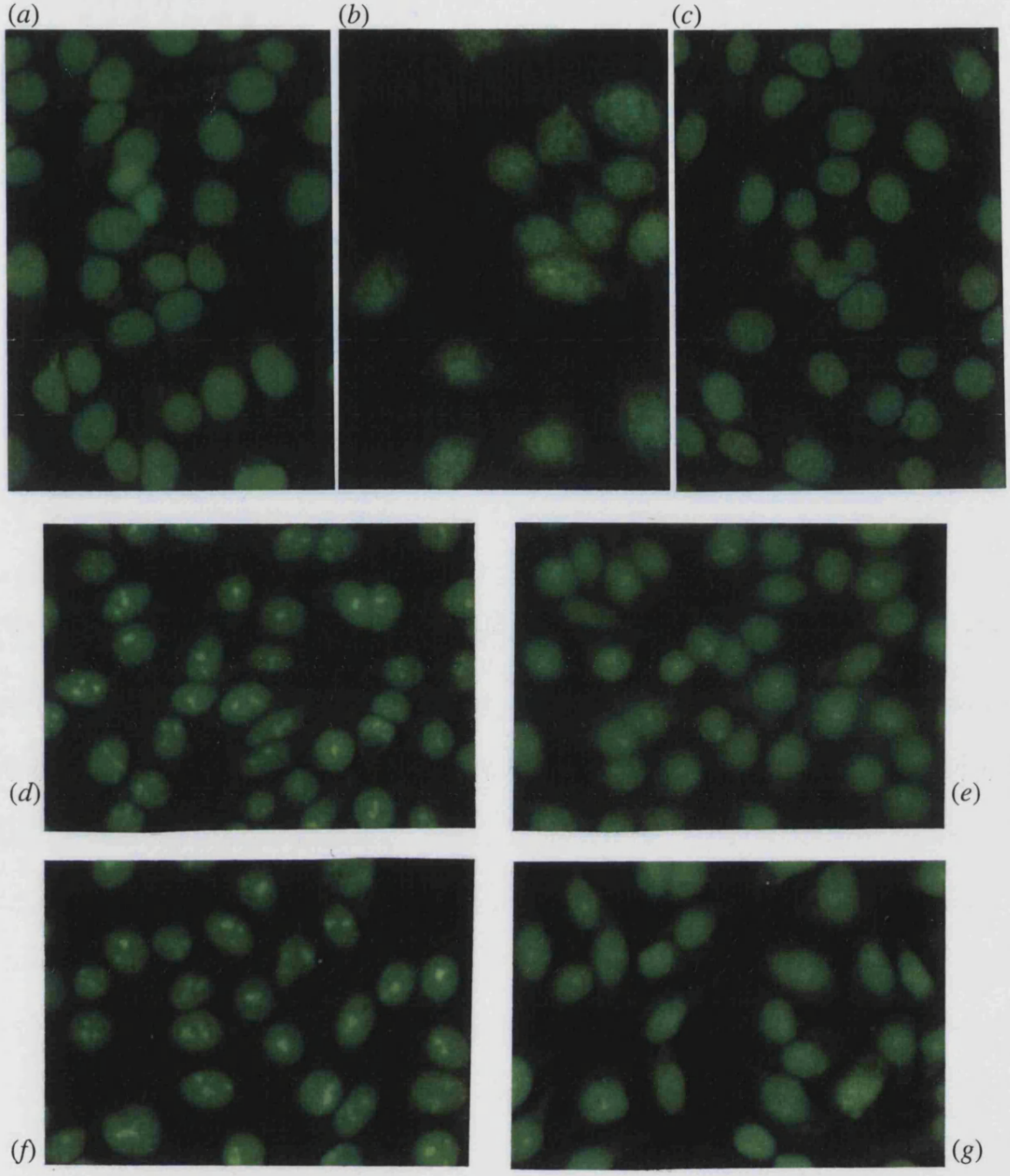


FIGURE 4.8 Indirect immunofluorescence patterns produced by sera from SSc patients which immunoprecipitated topoisomerase I and/or one or more RNA polymerase enzyme. (*a*) Serum EB, which precipitated RNA polymerases (RNAPs) I and III, produced a strong homogeneous nuclear pattern by indirect immunofluorescence (IF). However, IF patterns produced by anti-RNAP I/III sera were found to vary significantly from serum to serum (*cf.* Fig.3.4*a*). (*b*) Serum ML, which precipitated RNAPs I, II and III, produced a discrete speckled nuclear pattern by IF. (*c*) Meanwhile, a different anti-RNAP I/II/III serum (JMn) produced a homogeneous/fine speckled nuclear pattern, though nucleoli were distinguishable in some cells. (*c.f.* also Figs 3.4*b* and 3.5*a*). (*d*) The typical diffuse grainy speckled nuclear pattern with prominent nucleolar staining produced by anti-topoisomerase I (topo I) serum JeC: no RNA polymerases were immunoprecipitated by this serum. Patterns produced by sera from (*e*) anti-RNAP IIO/topo I, (*f*) anti-RNAPII/topo I and (*g*) anti-RNAP I/II/III/topo I sera (JP, Fo and PBu, respectively) were similar to that of the prototype anti-topo I serum. Patterns produced by sera with anti-RNAP III and anti-topo I/Ro/La specificities have been shown previously (see Figs 3.3*a* and 3.5*b*, respectively).

4.4 DISCUSSION

Prevalences of anti-RNAP antibodies, anti-topo I antibodies, and other anti-nuclear antibodies in SSc patients

In the present study, a total of 26 sera from 210 SSc patients (12%) were found to precipitate both RNAPs I and III. Thus, these results are in general agreement with previous findings in this area (36,144,183,185,255). The detection of anti-topo I antibodies in 44 (21%) SSc patients also compared well with previous data (36,52,53,327,357), and, furthermore, the incidences of anti-centromere, anti-Jo-1, and anti-U1 RNP antibodies reported here were also compatible with previous reports (20,36,52,53,143,177,202,261,343). Meanwhile, the frequency of anti-Ro antibodies (22; 10%) was higher than recently found in SSc patients by Bunn *et al.* (5%) (36). However, in addition to the method of immunodiffusion, we have included anti-Ro antibodies detected by the more sensitive technique of IP. Fujimoto *et al.* (104) have detected anti-Ro antibodies in 11% of SSc patients by immunodiffusion. Furthermore, using a sensitive ELISA technique, anti-Ro antibodies have been reported to occur in 37% of SSc patients by Bell *et al.* (17).

Subgroups of anti-RNAP antibodies were similar to those which have been reported before (36,144,183,255), i.e. a very small group with anti-RNAP III antibodies alone (in the present study, 2 (1%)), a larger group with anti-RNAP I/III antibodies (10; 5%), and a group capable of precipitating all three RNAPs (6; 8%). The additional detection of sera with antibodies which precipitated both RNAP IIO and topo I in the absence of RNAPs I and III (as found by Satoh *et al.* (299)) is discussed below.

Co-precipitation of autoantigens by SSc sera

Co-precipitation of RNA polymerases and topoisomerase I

Results presented here confirm the association of anti-RNAP IIO and anti-topo I antibodies in SSc, and extend the findings of Satoh *et al.* (299) to include Caucasian patients. The prevalence of sera with the anti-RNAP IIO/topo I antibody specificity found in Japanese SSc patients was 20%, while only 19 (9%) of the Caucasian SSc sera reported here were shown to co-precipitate RNAP IIO and topo I. However, when the data presented by Satoh *et al.* (299) are re-examined, and adjusted to include all SSc sera tested, regardless of race, then the figure of 12% is comparable with the present study. Why the incidence of anti-RNAP IIO/topo I antibodies should be skewed towards Japanese individuals in this way may have been a consequence of the small numbers involved in the study of Satoh *et al.* (299). Alternatively, genuine differences may exist between the two racial groups concerning anti-RNAP antibody expression. Racial differences in the incidence of particular autoantibodies have been reported before (6,189,228,280). A subsequent publication by the same group (297), however,

confirmed our original finding of sera with anti-RNAP IIO/topo I specificities in Caucasian SSc patients (126).

A further group of nine sera (4%) with autoantibodies to both forms of RNAP II, as well as to anti-topo I antibodies, has been identified in the present Caucasian group. As with the anti-RNAP IIO/topo I specificity, the majority of anti-RNAP II/topo I sera did not co-precipitate RNAPs I or III. The discrepancy between our study (126) and that of Satoh *et al.* (298,299) regarding the co-precipitation of RNAP IIA along with topo I and RNAP IIO in a minority (44%) of our anti-topo I/RNAP IIO sera was also resolved by their subsequent publication (297), which confirmed the existence of SSc sera with anti-RNAP II/topo I antibody specificities.

Importantly, Bunn *et al.* did not detect anti-RNAP IIO or anti-RNAP IIA antibodies in any of their anti-topo I sera (36). However, two points are in support of the existence of such an antibody profile in SSc sera. First, these sera were originally observed quite separately and independently in two different laboratories (136,298). Secondly, the present study demonstrated that these antibodies could be detected by IB techniques. In addition, difficulties in the visualization of RNAP IIo and IIa subunits were apparent in the study of Bunn *et al.* (36). Thus, it is possible that some of their anti-topo I sera did contain unidentified anti-RNAP II antibodies. This would not have affected the detection of anti-RNAP I or III antibodies, which they found with a very similar frequency (12%) as in the present study (13%). The three studies (36,128,299) are, however, in agreement concerning the mutual exclusivity of anti-RNAP III and anti-topo I antibodies (apart from one exceptional serum reported here).

Co-precipitation of RNAPs and other anti-nuclear antibodies by SSc sera

Another apparent difference between this study and that of Satoh *et al.* (299) is the relative frequencies with which certain other ANAs occurred in the anti-RNAP sera. A high incidence of Japanese anti-RNAP IIO sera also contained anti-Ro, anti-U1 RNP, anti-Ku or anti-Su autoantibodies (33, 6, 6 and 53% respectively). Of the eighteen anti-RNAP IIO/topo I and anti-RNAP II/topo I sera studied here, five (28%) contained anti-Ro, one (6%) contained anti-La, one (6%) contained anti-Jo-1, but none precipitated U1 RNP or the Ku antigen. We were not in possession of an anti-Su standard serum, and, consequently, were unable to detect and analyze the frequency of anti-Su antibodies in the present population (the Su autoantigen is precipitated as a triplet of 100-, 102-, and 200-kDa bands (300)). The figures for anti-Ro antibodies in the presence of anti-RNAP IIO antibodies compares well between the two studies, especially considering the small numbers involved. Furthermore, when the percentages calculated by Satoh *et al.* (299) are translated according to the total number of Japanese anti-RNAP IIO sera detected in their study (fifteen sera), the percentage of anti-RNAP IIO sera which co-precipitated either U1 RNP or the Ku antigen (6% and 6%, respectively) correspond to only one serum of each type. Therefore, the two studies are in accordance with one another on this point also, and, when considered together, imply that a minority (perhaps 1-5%) of

anti-RNAP IIO (±IIA) sera also co-precipitate U1 RNP, Ku, Jo-1 or La antigens, while a much higher proportion (~30%) of anti-RNAP IIO (±IIA) sera also precipitate the Ro antigen.

However, no ANAs were found in conjunction with anti-RNAP I or anti-RNAP III antibodies, a finding also supported by Bunn *et al.* (36). Therefore, in SSc, it appears that only those anti-RNAP-II-positive sera which also precipitate topo I contain anti-Ro antibodies, and, further, that anti-RNAP III antibodies and anti-Ro antibodies are mutually exclusive.

Other multiple autoantibody specificities

The presence of other multiple autoantibody specificities in some SSc sera was also of interest. Six sera (3%) were found to precipitate both topo I and the Ro antigen in the absence of anti-RNAP antibodies. In four of these cases, two separate lines of identity had been observed by immunodiffusion techniques (Table 4.4), supporting the existence of distinct antibodies. Meanwhile, only one serum (0.5%) was found to contain both anti-topo I and anti-U1 RNP antibodies (Table 4.5). An earlier report by Sato *et al.* detected five sera (3%) with both these specificities in their cohort of 176 SSc and 30 MCTD patients (189). However, the overall prevalence of anti-U1 RNP antibodies was in any case rather high in their study (25%), as has previously been demonstrated in Japanese SSc patients. None of the other SSc-specific antibodies, i.e. ACAs, anti-Th RNP, -Pm-Scl or -U3 RNP antibodies were found to be co-precipitated with any of the RNAPs detected here. Also, of the 41 ACA-positive sera tested here, none was found to contain anti-topo I or anti-RNAP antibodies. Thus, these results are in accordance with previous reports (255).

Together, these results support the existence of three main groups of SSc sera, each characterized by the presence of a particular, mutually exclusive, SSc-specific autoantibody (ACAs, anti-topo I antibodies or anti-RNAP III antibodies), as suggested by Bunn *et al.* (36). Anti-topo I antibodies are frequently accompanied by antibodies to Ro (± La) and/or RNAP IIO (± IIA). Anti-RNAP III antibodies are usually accompanied by antibodies recognizing RNAP I, and sometimes also by anti-RNAP II antibodies. Meanwhile, ACAs tend to occur alone.

Clinical associations of autoantibodies in SSc

Disease subtype associations

Results presented here confirm the very close association between the presence of ACAs and limited disease, and the association between the presence of each individual anti-RNAP antibody and diffuse disease is also in support of previous data. In the present study, however, the anti-RNAP I/II/III group had a highly significant association with dc-SSc, while the anti-RNAP I/III group did not.

Meanwhile, the association of anti-topo I antibodies with diffuse disease has not been confirmed. Several factors could have led to a majority of our anti-topo I-positive patients having lc-SSc, including slightly different clinical interpretations of diffuse and limited SSc. As mentioned, clinical associations of the three main SSc antibody groups, i.e. ACAs, anti-topo I antibodies and anti-RNAP antibodies, have been studied simultaneously on relatively few occasions, while anti-topo I and ACAs have often been compared in the same patient population. The present results confirm that the anti-topo I group is associated with a significantly higher risk of dc-SSc compared to the ACA group, while the anti-RNAP III-positive group is shown to have an even greater incidence of diffuse disease, significantly higher than either the anti-centromere- or the anti-topo I-positive groups. When Giordano *et al.* (114) divided their 90 SSc patients into six groups based on the extent of cutaneous involvement, they found that, while ACAs were associated with the limited cutaneous groups (Groups 1 and 2), anti-topo I antibodies were associated with both the diffuse and the intermediate subsets (Groups 4 and 5), while the diffuse subsets (Groups 5 and 6) were mainly composed of sera which displayed ANoA patterns by IF (these were not identified, but may have included anti-RNAP I-positive sera). Furthermore, Steen *et al.* (255) showed that, amongst dc-SSc patients, only 27% had anti-topo I antibodies, while 45% had anti-RNAP III antibodies.

The results of the present study would suggest that the three main SSc-specific antibodies are each distinguished by different degrees of cutaneous involvement, with members of the ACA group generally having the mildest degree of cutaneous spread, patients in the anti-RNAP III group frequently having the most diffuse form of the disease, and members of the anti-topo I group tending to have an intermediate pattern (and/or risk) of cutaneous manifestations.

Renal involvement

The association of renal involvement with anti-RNAP antibodies was not confirmed in the present study. There is evidence that the selection of patients for the SSc family study was biased against the inclusion of patients with renal involvement (Dr N.J.McHugh, pers.comm.). Furthermore, we have reported separately on our own anti-RNAP-positive patients (which are included here alongside patients from the SSc family study), and have shown a significant association of anti-RNAP antibodies with the presence of renal involvement (129).

Meanwhile, a significant association between renal involvement and the presence of anti-La and/or anti-Ro antibodies has been demonstrated here. However, some of these particular sera contained other ANA specificities, and the numbers involved were relatively small. Therefore, the existence of a link between anti-Ro (± La) antibodies and renal involvement would require further confirmation: this is one aim of our future research.

In the present study, the anti-topo I group had a significantly increased rate of renal involvement compared with the ACA group. Our finding of 29% renal involvement in

anti-RNAP I/II/III patients was comparable with that reported by Kuwana *et al.* (185) and by Bunn *et al.* (36) (43% and 33%, respectively). However, our anti-topo I-positive patients had a somewhat higher rate of renal disease (20%) than their anti-topo I-positive groups (6% and 3%, respectively), while the incidence of renal disease in our ACA-positive patients (2%) was similar to that found by Kuwana *et al.* (185) (0%). A comparable study by Steen *et al.* (255) reported renal involvement in 33% of their anti-RNAP III-positive patients and 10% of their anti-topo I-positive patients.

While our criteria for renal involvement may have been less stringent than in other studies, these data provide further evidence of distinctive clinical patterns being associated with each of the three autoantibody groups discussed above.

Pulmonary involvement

In the present study it was shown that, whether occurring separately or together, anti-topo I antibodies and anti-Ro antibodies were each significantly associated with pulmonary involvement. Although most studies have concentrated on the prognostic significance of SSc-specific antibodies in SSc, an association between pulmonary involvement and anti-Ro antibodies in SSc has been reported previously (261). Again, an apparent link between the anti-topo I antibody response and the anti-Ro antibody response is implied by these results. In view of the significant association of anti-Ro antibodies with both renal involvement and lung involvement, it appears possible that anti-Ro antibodies are of relevance to SSc pathogenesis, particularly in the context of anti-RNAP II and/or anti-topo I antibodies.

Autoreactive subunits of RNA polymerases

The present findings indicate that autoreactive epitopes are located on RNAP complexes which are distinct and separate from those on topo I. First, all anti-RNAP sera still precipitated the same RNAPs in the absence of topo I. Secondly, IB studies indicated that anti-topo I sera recognized the 100-kDa topo I band, whether or not the sera also had anti-RNAP II or RNAP IIO autoantibodies. Thirdly, anti-RNAP IIO autoantibodies were detected by recognition of the largest, phosphorylated subunit (RNAP IIo; 240 kDa). In further support of this conclusion, it should be mentioned that the specificities of anti-topo I antibodies detected by IP and immunodiffusion were independently confirmed by Dr J. Whyte using a topo I functional inhibition assay, as described previously (227). Dr Whyte reported that all sera with anti-topo I antibodies by IP and immunodiffusion were able to inhibit topo I, including sera with anti-RNAP II/topo I, anti-RNAP IIO/topo I, and anti-RNAP I/II/III/topo I specificities, some of which had also precipitated the Jo-1, Ro, and/or La antigens (Table 4.3; (127)). Furthermore, as indicated on Table 4.3, two anti-RNAP II/topo I/Ro sera recognized subunits IIo and IIa on immunoblots, and also produced a positive result by immunodiffusion, producing lines of identity with both prototype anti-topo I sera and with anti-Ro prototype sera. Anti-RNAP antibodies are not detected by

immunodiffusion. These results suggest that antibodies in these sera recognized separate epitopes on all three antigens.

Since the unphosphorylated IIa subunit of RNAP II (220 kDa) was also recognized by the majority of sera which had precipitated RNAP IIA, but never by sera which precipitated only RNAP IIO, it was further concluded that at least two different sites on the largest subunit of RNAP II are recognized by SSc sera, and that one of these sites is unique to the phosphorylated (IIO) form.

In the current report, the autoreactive subunits of RNAPs I and III were not identified by IB, although autoradiographs showed the presence of constituent subunits on affinity purified preparations transferred onto nitrocellulose. Previous studies have shown specific recognition of particular RNAP I and III subunits by anti-RNAP sera using slightly different methods. Using a HeLa-cell RNAP-enriched fraction, Kuwana, Kaburaki & Mimori (183) showed that one or other of the two subunits common to all three RNAPs (25 and 42 kDa) are recognized by most anti-RNAP I/III and anti-RNAP I/II/III sera (183). Although the extract used contained all three classes of RNAP, no serum reacted with any RNAP II-specific subunits. Okano *et al.* used a biochemical method to purify HeLa-cell RNAP III. The 138-kDa subunit, unique to RNAP III was recognized not only by anti-RNAP III sera, but also by anti-RNAP I/III and anti-RNAP I/II/III sera (255). All three groups also recognized the ~25-kDa subunit mentioned above.

In the present study, despite repeated attempts, antigenic determinants specific to RNAP I or RNAP III were not detected, possibly due to less sensitive methods of detection than those used by others (183,255). The cell type used is an unlikely explanation since RNAPs are ubiquitous and their structures well conserved. One further possibility is that autoreactive epitopes on one or more RNAPs are present only in the native conformation, especially considering that anti-RNAP III autoantibodies are able to inhibit transcription by native RNAP III (183).

Meanwhile, in the study of Hirakata *et al.* (144), the 220- and 145-kDa subunits of RNAP II were isolated from calf thymus and used as substrates in IB studies with anti-RNAP I/II/III sera. The 220-kDa subunit was recognized by 9/13 of these sera and the 145-kDa subunit was recognized by 11/13. However, also mentioned in this paper was the observation that 12/13 of the same sera could immunoprecipitate the 23-kDa subunit, common to all three RNAPs, thus confirming the findings of Okano *et al.* (255), and Kuwana *et al.* (183) mentioned above.

Therefore, the present data, together with the results of others, suggest that, while anti-RNAP II/topo I sera and anti-RNAP IIO/topo I sera only recognize RNAP subunits which are specific to one or both forms of RNAP II, anti-RNAP I/II/III sera also recognize subunits common to all three RNAPs, and also recognize RNAP III-specific subunits.

4.5 CONCLUSIONS

There are two different pathways for the production of anti-RNA polymerase II antibodies in SSc

It is of great significance that no sera have been reported that precipitate only RNAPs I, IIO and III, while anti-RNAP IIO/topo I sera are relatively frequent. Furthermore, sera which precipitate only RNAP I, RNAP II, RNAP IIO, RNAPs I and II, or RNAPs II and III appear to be exceptionally uncommon in SSc. These data may indicate that, in most anti-RNAP-positive SSc sera, the anti-RNAP response begins with a breakdown of tolerance to one of a very small number of key autoantigens, and, furthermore, that the identity of the 'inciting' autoantigen, in turn, determines a particular, characteristic pathway of epitope spread. The apparent mutual exclusivity of anti-RNAP III and anti-Ro antibodies in SSc may provide further clues concerning the patterns of epitope spread occurring in particular SSc patients.

Specifically, when considered together, the various aspects of the anti-RNAP II antibody response strongly suggest that two different immune response pathways can lead to the production of anti-RNAP II antibodies in SSc. Indeed, in the case of the anti-RNAP I/II/III group, the model suggested by Hirakata *et al.* (142,144) favours the initial production of anti-RNAP I or anti-RNAP III antibodies, then, due to recognition of the shared 25-kDa subunit, the spreading of the immune response to include the RNAP II enzyme (144). This could be followed by eventual intramolecular epitope spreading within RNAP II which would often include antibodies specifically recognizing the IIo subunit and/or the IIa subunit. The present data are wholly compatible with this account. Significantly, one of our anti-RNAP I/II/III sera recognized only subunit IIo on IBs, although the same serum was capable of precipitating both RNAP IIO and RNAP IIA equally, presumably on account of common epitopes, possibly located on the 25-kDa subunit.

Meanwhile, the present data suggest that anti-RNAP IIO/topo I sera and anti-RNAP II/topo I sera do not recognize subunits common to all three RNAPs. Furthermore, anti-RNAP IIO/topo I sera do not recognize epitopes present on both RNAP IIO and RNAP IIA, since only RNAP IIO is precipitated by this particular group of sera, and only subunit IIo was ever recognized by these sera on IBs. Therefore, in the anti-RNAP IIO (± IIA)/topo I group, the immune response appears to begin with either topo I or RNAP IIO, with subsequent spread to include RNAP IIA in some cases. It therefore follows that, in the anti-RNAP I/II/III group, the original inciting antigen is highly unlikely to have been RNAP II: the recognition of RNAP II in this group appears to have resulted from epitope spread via a subunit shared with RNAPs I and III. Meanwhile the anti-RNAP II response in the anti-RNAP IIO (± IIA)/topo I group seems most likely to have resulted from the presentation of cryptic epitopes of either topo I or RNAP IIO or both. These conclusions suggest the presence of alternative pathological conditions in the two

groups of patients which characteristically lead to the production of anti-RNAP III antibodies on the one hand and anti-topo I antibodies on the other.

Furthermore, based on the present data, it would appear that, although the anti-RNAP I/III antibody response may spread to include a common subunit found in RNAP II it does not subsequently spread to include topo I, while the anti-RNAP II/topo I antibody response does not appear to spread to include RNAPs I and III. Such discretionary epitope spreading, occurring in one direction but not in the other, may be explained by the nature of antigen processing by B cells, which is known to be affected by the specificity of the particular B cell concerned: since the antigenic site bound by the Ig molecule is protected from proteolysis, different peptides can be generated from the same antigen by B cells, depending on their particular epitope specificity (212).

A consequence of the production of anti-RNAP II antibodies by two distinct pathways regards HLA associations with disease, i.e. the context of antibody production may be important to its HLA associations. If, for example, a particular autoantibody is produced via processing of a particular cryptic epitope, then its HLA associations may be different to those of an antibody with the same specificity which has been produced as a result of processing of non-cryptic antigens during epitope spread, as is suggested by the recent report of Frank *et al.* (97).

There are three main clinical and serological groups of SSc patients

Based on a detailed analysis of HLA associations of SSc-specific autoantibodies (86), together with their previous results (36), it has been suggested by Fanning *et al.* (86) that the three main serologically defined subgroups of SSc patients actually represent patients with three different diseases.

The present study would support this hypothesis, but with the added point that the anti-topo I group is also associated with the occurrence of anti-Ro and/or anti-RNAP II antibodies. It seems possible that, in the context of this particular subset of SSc patients, the three antigens, RNAP II, topo I and Ro are embroiled in a sequence of events which results in a breakdown of tolerance to one, two or all three molecules. Significantly, both RNAP II and topo I (but not RNAP I, RNAP III or centromere proteins) were among the SSc antigens found to be uniquely susceptible to metal ion-catalyzed fragmentation under conditions of oxidative stress by Casciola-Rosen *et al.* (46). Since metal ions colocalize with these SSc antigens, such fragmentations may occur *in vivo* during ischaemic reperfusion. As they have pointed out, the array of autoantibodies found in a particular autoimmune syndrome represents the 'immunological memory' of pathological events (288). Thus, the subset of patients with anti-RNAP II and/or topo I antibodies described here may well correspond to the particular subgroup of SSc patients proposed by Casciola-Rosen *et al.* (46) to have unique access to RNAP II and topo I antigens in a particular, novel fragmented form. In their paper, it was hypothesized that these modified proteins permitted the presentation of cryptic epitopes to T cells, resulting in a breakdown of tolerance to these antigens. Thus, the findings of

Casciola-Rosen *et al.* could explain the mutual exclusivity of the anti-topo I-positive group from the ACA group and the anti-RNAP III-positive group. The present results are in keeping with the existence of other, distinct pathological mechanisms which may underlie the breakdown of tolerance to RNAP III, and still others to the breakdown of tolerance to CENPs, as suggested by Rosen *et al.* (143). Were this shown to be the case, the separate disease model of Fanning *et al.* (86) would be inherently supported.

However, the Ro autoantigen was not found to be fragmented under conditions of oxidative stress by Casciola-Rosen *et al.* (46), and, consequently, the pathological processes underlying the co-existence of anti-Ro, anti-topo I and anti-RNAP II antibodies in some SSc patients are less clear. On this point, it is of interest that anti-RNAP II and anti-Ro antibodies are common in SLE sera (297), while reports of anti-topo I and other anti-RNAP antibodies in SLE are very rare (141). A pathogenic link between SLE and the subgroup of SSc characterized by the production of anti-Ro and/or anti-RNAP II antibodies is worth considering. However, an alternative possibility is suggested by the recent discovery that anti-EC antibodies are capable of inducing EC apoptosis (29). The anti-Ro subgroup may represent those SSc patients in whom excessive EC apoptosis takes place: such events may involve the production of blebs by the apoptotic ECs analogous to the blebs which occur during apoptosis of keratinocytes, and which were proposed by Rosen *et al.* to be involved in the production of anti-Ro antibodies in SLE patients (see Section 1.7; (47)). High levels of EC apoptosis occurring in the context of the inflammatory perivascular micro-environment of SSc may thus lead to the production of anti-Ro antibodies, especially considering the occurrence of complement deficiencies in a proportion of SSc patients.

Further groups of SSc sera include those characterized by the presence of antibodies recognizing U3 RNP, Th RNP, Pm-Scl, Jo-1 and U1 RNP antigens. Based on previous results (127,251,252,294), together with the present data, it would appear that patients with each of these specificities also form distinct groups, which, in the majority of cases, are mutually exclusive. However, the proportions of SSc sera which contain each of these antibodies is relatively small. Here again though, distinct molecular processes may be at work, such as have been suggested by the induction of anti-fibrillarin antibodies in mice following treatment with mercury (151,152). It has been proposed that mercury modifies the structure of fibrillarin, forming a neoepitope that is immunogenic (120,270).

Distinct pathological processes in the three groups of SSc patients are responsible for the characteristic symptoms, for the modification of particular autoantigens, and, consequently, for the production of different autoantibodies

An important factor which contributed to the highly significant association of the anti-RNAP I/II/III group with diffuse disease appears to have been the presence of anti-RNAP II antibodies, since the anti-RNAP I/III group had a lower incidence of diffuse disease, which was not significantly different from the remaining group. Meanwhile,

the incidence of dc-SSc found in both the anti-RNAP II/topo I group, and in the group of patients who had anti-topo I antibodies in the absence of anti-RNAP antibodies was identical, and rather low. Thus, our data suggest that the presence of anti-RNAP II antibodies contributes to the high incidence of dc-SSc in the anti-RNAP I/II/III group, but that a similar effect does not occur in the anti-RNAP II/topo I group. Although the numbers involved are too small to allow definitive conclusions, it appears that the presence of anti-RNAP II antibodies *per se* does not lead to an increased incidence of dc-SSc. Rather, the anti-RNAP I/III antibodies and the anti-RNAP II antibodies seem, in effect, to be acting synergistically to produce dc-SSc. An alternative, and more plausible, possibility is that the context in which anti-RNAP II antibodies are produced is a key aspect of their clinical significance. This would be an important observation, since it links disease expression with events leading to the production of particular cryptic epitopes of an autoantigen, rather than with the presence of a particular autoantibody as such. With regard to patients with anti-RNAP III antibodies, one distinct possibility is that the anti-RNAP I/III group and the anti-RNAP I/II/III group are actually a single subgroup of patients at a different stage of their disease, and that those anti-RNAP I/III patients who currently have 'limited' disease are likely, subsequently, to develop diffuse skin involvement, and, in the presence of appropriate HLA alleles, anti-RNAP II antibodies. The validity of this model could be tested by long-term serial measurement of anti-RNAP antibodies in SSc sera by IP assays.

Thus, although definitive proof was not obtained, several points suggest that fundamental differences exist in anti-RNAP II antibody production by different groups of SSc patients, and that these are dependent on the context of the anti-SSc-specific antibody which they accompany. This could result from distinct sets of cryptically presented antigens, produced in the context of different pathological processes.

It should be mentioned that the IF results also supported this model, since all topo I-positive sera showed a similar pattern of fluorescence, regardless of the presence or absence of RNAP II, while anti-RNAP I/II/III and anti-RNAP-I/III sera showed a rather different pattern, which was somewhat variable, presumably depending on the relative titres of the various antibodies recognizing the different component subunits of the three different RNAPs.

Concerning the three main groups of SSc patients: based on the available data, the most likely model is for the existence of three distinct disease processes each having certain key pathological features. In turn, each pathological state leads to the production of a distinctive set of modified autoantigens and, consequently, to the production of particular SSc-specific antibodies. Thus, identification of the original inciting antigen recognized by SSc sera may be the key to the subgrouping of SSc patients, as implied by the work of Casciola-Rosen *et al.* (47-49), Utz *et al.* (347), and Casiano *et al.* (50) in SLE, and, most particularly, the reports of Rosen *et al.* concerning the unique, oxidative stress-induced cleavage of certain SSc autoantigens (46,288).

CHAPTER 5

RESULTS, PART 3

Characterization of novel autoantigen systems in the relatives of systemic sclerosis patients

5.1 INTRODUCTION

The identification of SSc-associated antibodies, together with the detection of undefined autoantibody specificities, in sera from 62 SSc patients and their 239 family members was described previously ((127); Chapter 3). Following IP of ^{35}S-methionine-labelled proteins from K562-cell extracts, and separation by SDS-PAGE, twenty-nine samples (from eight SSc patients (12.9%), 20 SSc-free relatives (9.3%) and one spouse (4.2%)) produced bands of unknown specificity, including sera from four probands who also had defined SSc-specific autoantibodies. Many of the unidentified IP bands were weak, but strong unidentified bands were seen with samples from six SSc patients (9.7%), twelve SSc-free relatives (5.6%) and one spouse (4.2%). A strong band at ~115 kDa was produced by three SSc patients and seven SSc-free family members. This included the spouse (BD2) and three sisters (BD3, BD5 and BD7) of a proband (BD1) with anti-U1 RNP antibodies. Although a proportion of 120 normal sera also showed the presence of unidentified bands by IP (seven; 5.8%), only three were strong (2.5%), and none was found to precipitate a band of ~115 kDa.

Just as in SSc, many of the unidentified autoantigens recognized by sera from the first-degree relatives of SSc patients appeared to be located in the nucleolus, as indicated by the significantly increased incidence of IF-ANoAs in blood-relatives compared with controls. It was suggested that these undefined ANoAs may have resulted from some form of genetic or environmentally induced abnormality, present in both SSc patients and in some of their relatives, which worked to make the nucleolus a target for the immune response. It was further proposed that, along with this abnormality, additional pro-pathological factors (genetic or environmental) occurred in SSc patients and that these were responsible for the development of the complete disease syndrome, and, also, for the generation of particular sets of modified autoantigens. In turn, these caused the subsequent generation of SSc-specific autoantibodies in the proband. An alternative possibility was also mentioned: that the unidentified autoantigens represent proteins which are early participants in the aetiopathogenesis of SSc. Again, additional genetic or environmental factors, present only in those destined to develop SSc, may accompany the propagation of these early events to the development of the complete disease syndrome, together with the particular autoantibodies characteristic of SSc.

In the present study, eight of these "anti-115-kDa" sera were further investigated, to determine whether the same protein was being precipitated in all cases, and to isolate, characterize and identify the 115-kDa autoantigen/s. The immunoprecipitated proteins were first compared by SDS-PAGE analysis on 10%- and 7.5%-polyacrylamide gels. Relationships between the antigens precipitated by the study sera were then pursued by a combination of antigen depletion studies, and IB experiments using affinity purified autoantigens. Indirect immunofluorescence studies were also carried out in order to describe clearly the subcellular distribution of the 115-kDa autoantigens. Finally,

immunoaffinity column chromatographic techniques were performed, with the aim of purifying some of the 115-kDa autoantigens for subsequent amino-acid sequencing and identification.

During the early stages of this work, it was suggested (Dr N.J. McHugh, pers. comm.) that one or all of these precipitated proteins could be PADPRP, which has a molecular weight of 116 kDa. This enzyme catalyzes the repetitive transfer of ADP-ribose monomers from NAD^+ to a particular range of nuclear proteins, most of which are directly involved in either nucleic acid metabolism or maintenance of chromatin structure, including histone proteins H1 and H2B, topo I, RNAP II and PADPRP itself (240,272). In some circumstances the enzyme is thought to be responsible for triggering stress-induced apoptosis (246), following excessive DNA fragmentation, and the consequent stimulatory signal of intracellular NAD^+ depletion (19). However, under conditions where there is less severe DNA damage, PADPRP activity may also function to promote DNA strand rejoining, and subsequent cell recovery (219,302).

The synthesis of poly(ADP-ribose) has been found to be decreased in family members of SLE patients, as well as in SLE patients themselves ((131); for review (309)). Further, sera from SLE patients (and a lower frequency of SSc patients) have been found to contain anti-poly(ADP-ribose) antibodies (72,81,148). Antibodies recognizing the PADPRP enzyme itself have also been reported in a variety of patients with CTDs (including SSc), particularly those with SLE and/or SS (240,369). In addition, aged mice with an inactivated PADPRP gene have been found to be prone to the spontaneous development of epidermal hyperplasia (355). To test the hypothesis that some or all of the eight study sera were precipitating PADPRP, an anti-PADPRP monoclonal antibody (MAb) was included in some of the antigen depletion experiments (kind gift of Dr Sylviae Muller, Institut de Biologie Moleculaire et Cellulaire, Strasbourg, France).

5.2 MATERIALS AND METHODS

Clinical details and serology

Details regarding the eleven individuals whose sera were found to immunoprecipitate antigens of ~115 kDa from K562-cell extracts in the SSc family study are summarized on Table 5.1. All four patients were female, and all had the limited form of SSc. Additional unidentified bands were precipitated by two of the SSc sera, and three of the SSc patients had defined autoantibody specificities (Table 5.1*a*). None of the seven family members had a defined CTD, but three displayed symptoms of RP (43%: *c.f.* 26% total of SSc-free family members in the SSc family study had RP) (Table 5.1*b,c*).

All seven anti-115-kDa sera from family members were selected for inclusion in the present study, together with one serum from an SSc patient (Table 5.2). The original serum samples were used (see Chapter 3), which had been kept stored at -20°C. Apart

from the BD family (Table 5.2: Family 1 (F1)), where sera from the husband and three sisters of the SSc patient each precipitated a 115-kDa band, two of the sera were from mothers of SSc patients (F2 and F3), and one was from the son of an SSc patient (F5) (Table 5.2).

Immunodiffusion

All eight study samples had been screened by Ouchterlony double immunodiffusion as part of the SSc family study (Chapter 3; Table 5.1), and these tests were not repeated.

Immunofluorescence

Each of the eight study samples had been included in IF tests as part of the SSc family study, using HEp-2-cell slides from Biodiagnostics Ltd (Chapter 3; Table 5.1). However, our diagnostics laboratory had recently started using a superior brand of commercially prepared HEp-2 slides from Light Diagnostics Ltd (Mrs J.Dunphy, pers. comm.), and it was decided to repeat IF testing using the new slides. The opportunity was also taken to titre the sera. The usual method was used (Method 2.3.2), with each of the eight study sera being tested at the following dilutions: 1/40, 1/160, 1/640 and 1/2560. In addition to antinuclear and antinucleolar IF, anti-cytoplasmic staining patterns were recorded. Antinuclear, antinucleolar and anticytoplasmic IF intensities were each scored as negative (-), very weak (+/-), weak (+), moderate (++), strong (+++) or very strong (++++). A number of sera with known autoantibody specificities were also tested, to serve as controls, and these included other members of the BD family (F1).

Comparison of immunoprecipitated proteins

Each of the eight study sera had been included in protein IP studies as part of the SSc family study (see previous Fig. 3.20). During the present study, each of these sera was again subject to protein IP techniques, using a single batch of ^{35}S-methionine-labelled K562-cell extract (Method 2.3.3*a*). Again, other members of the BD family (F1) were included, along with prototype sera of known autoantibody specificity. Resulting immunoprecipitated proteins were separated on a 10%-polyacrylamide gel, dried, and autoradiographed as usual at two different time points. This allowed a subjective comparison of the relative band intensities produced by the eight sera to be made, which is influenced by (i) the titre of the serum, (ii) the avidity of the antibody, (iii) the relative abundance of the precipitated antigen in the cell extract, (iv) the molecular weight of the antigen, (v) the percentage methionine content of the antigen, and (vi) the turnover rate of the antigen *in vivo*.

Duplicate IP samples were then separated on a 7.5%-polyacrylamide gel, to compare the molecular weights of the different proteins. When proteins of this molecular weight are being studied, small differences in electrophoretic mobility are more readily apparent in lower percentage gels.

Antigen depletion of K562-cell extracts

Similar IP experiments were then performed using aliquots of radiolabelled K562-cell extract which had been precleared of antigenic particles recognized by (i) serum BD5 (F1: Sister 2), (ii) serum BD2 (F1: Husband), and (iii) normal control serum (Method 2.3.3*c*). Four cycles of depletion were carried out to ensure removal of all traces of antigen. Along with the eight study sera, the anti-PADPRP MAb, an anti-U1 RNP prototype serum, and sera from the remaining members of the BD family (F1) were included amongst the test sera in these experiments.

Immunoprecipitation of ribonucleoproteins

Since serum from BD1 (F1: Patient) was known to contain anti-U1 RNP antibodies, it was considered possible that sera from other members of this family may contain antibodies which precipitated one or more UsnRNPs. Unlabelled cell extracts were prepared, and the eight study sera were subjected to RNA-IP techniques along with appropriate standards (Method 2.3.3*b*). The PCA-extracted samples were run on urea-polyacrylamide gels, which were silver stained and dried, in order to detect any RNAs associated with the unidentified autoantigens.

Immunoblotting of affinity purified autoantigens

Each of the eight study sera was used to affinity purify autoantigens from cell extracts by scaled-up IP techniques using ^{35}S-methionine-labelled K562-cell extracts (Method 2.3.3*d*). Following separation by SDS-PAGE, purified polypeptides were transferred onto nitrocellulose sheets. Immunoblotting studies were then performed using each purified protein extract as an antigen source. Each of the eight study sera was then tested against each of the eight purified antigen preparations as usual, with prototype sera of known autoantibody specificity being included for comparison. Again, other members of the BD family (F1) were also included. Immunoblots containing radiolabelled proteins were then subjected to autoradiography to reveal the positions of transferred polypeptides.

Similar experiments were then carried out using affinity purified antigens prepared using unlabelled K562-cell extract, in which case the quantities of Protein-A Sepharose, IPP buffer, serum, and cell extract used were further increased (3 x those stated in Method 2.3.3*d*): this allowed a greater concentration of purified antigen to be contained on each of the final blotting strips. In these experiments only two sera, BD2 (F1: Husband) and BD5 (F1: Sister 2), were used to purify antigens, while all eight study sera, together with controls, were included as test sera.

Purification of autoantigens by immunoaffinity column chromatography

Three sera from the present study (BD2 (F1: Husband), BD5 (F1: Sister 2) and MD9 (F5: Son)) were selected for immunoaffinity column chromatography trials. These preliminary tests were carried out by preparing three mini-columns, each containing a

2-ml bed volume of Protein-A Sepharose CL-4B beads covalently linked to antibodies from the relevant serum. A 2-ml normal-serum pre-column was also constructed. The protocol described in Method 2.3.4 was followed, with all volumes being scaled down by a factor of 2.5. Following application of the low-pH glycine buffer, fractions of eluate were collected and tested for the presence of 115-kDa bands by silver-staining techniques. Any successfully eluted antigens were further purified by de-salting. Purified proteins were then subjected to SDS-PAGE using a 10%-polyacrylamide gel, and transferred to PVDF membranes. Coomassie blue staining of blots revealed the position of isolated proteins, which were sent for amino-acid sequencing.

Based on these preliminary results, a full-sized column was later constructed, containing a 5-ml bed volume of Protein-A Sepharose CL-4B beads covalently coupled to serum antibodies from a fourth study serum, BD3 (F1: Sister 1) (Method 2.3.4).

5.3 RESULTS

Immunofluorescence

Results obtained using the two different brands of HEp-2-cell slides can be compared on Tables 5.1 and 5.2. In most cases, slightly different IF patterns and/or intensities were recorded when the same serum sample was tested on the two different types of slides. While the majority of differences were of a single gradation of intensity (e.g. the speckled nucleolar staining produced by serum from family member BE6 was strong with slides from Biodiagnostics Ltd (Table 5.1), but only moderate with slides from Light Diagnostics Ltd (Table 5.2)), occasionally differences were more substantial (e.g. with Biodiagnostics Ltd slides, serum from SSc patient LK1 showed a strong discrete course speckled nuclear pattern, nucleolar sparing; with slides from Light Diagnostics Ltd, however, a moderate grainy speckled staining pattern of both nucleus and nucleolus was observed).

Little uniformity was apparent between the eight study sera regardless of which slides were used (Tables 5.1 and 5.2). However, close examination reveals that two sera from F1 (BD3 (Sister 1) and BD5 (Sister 2); Tables 5.1 and 5.2) produced a very similar pattern of nuclear IF, while the other two F1 sera shared a different, predominantly cytoplasmic pattern (BD2 (Husband) and BD7 (Sister 3); Table 5.2). All four sera from F1 had a high antibody titre (Table 5.2). The patterns produced by sera from F1 in the SSc family study (using slides from Biodiagnostics Ltd) are shown in Fig. 5.1. For comparison, results obtained with some of the study sera when using slides from Light Diagnostics Ltd are shown in Fig. 5.2*b,c,d*. Prototype sera which had previously been tested on Biodiagnostics Ltd slides were also tested using the new slides (Fig. 5.2*a,e,f,g*): again, slightly different patterns were sometimes produced by the two types of slides when the same serum was tested. On the whole, patterns

produced by slides from Light Diagnostics Ltd were more distinctive, and were easier to read.

Comparison of immunoprecipitated proteins

When the eight study sera were immunoprecipitated and run on 10% SDS polyacrylamide gels (Fig. 5.3*a*), no obvious differences in electrophoretic mobilities were apparent. However, subjective differences between the band intensities of proteins precipitated by the study sera were clear. These were carefully judged using two different autoradiographs, one of which was exposed for twice as long as usual (Fig. 5.3*b*). The large amounts of antigen apparently precipitated by sera from BD3 (F1: Sister 1, lane 8) and BD5 (F1: Sister 2, lane 5) were striking, and, in both cases, the bands were rather fuzzy. This phenomenon is known to occur with phosphorylated proteins, due to a number of variably dephosphorylated breakdown products. The U1 RNP-positive proband of F1 (BD1) was also included on this gel (lane 9). By comparing with previous Fig. 3.6, it seems likely that most of the study sera recognize antigens which are present in a fairly high copy number per cell (i.e. the strength of the bands resembles bands produced by U1 RNP and other ANAs rather than the ANoAs, which are about 10 x less plentiful per cell).

When the same experiment was conducted using an 8% SDS-polyacrylamide gel, relative band mobilities remained indistinguishable between the eight study sera (Fig. 5.4).

Immunoprecipitation of ribonucleoproteins

The appearance of the silver-stained urea-PAGE-separated samples is shown in Fig. 5.5. Although the standard sera precipitated the RNA species associated with the particular antigen concerned, no members of F1 were found to precipitate any RNA species. However, serum from BD1 (F1: Patient) was confirmed positive for anti-U1 RNP antibodies, and U2 RNP was also found to be precipitated by this serum, as indicated by the presence of U2 RNA on the silver-stained gel (Fig. 5.5, lane 3).

These findings indicate that none of the anti-115-kDa sera tested recognized any RNPs.

Antigen depletion of K562-cell extracts

A radiolabelled extract was shown to contain antigens precipitated by all the test sera (Fig. 5.6*a*, lanes 2-9). Then, the extract was specifically depleted of antigens recognized by antibodies contained in serum from BD5 (F1: Sister 2) by successive incubations with BD5-antibody-coated Protein-A Sepharose beads (Fig. 5.6*b*, lanes 1-5). Meanwhile, a normal serum did not adsorb out any 115-kDa antigens (Fig. 5.6*b*, lanes 12-16), including the 115-kDa antigen precipitated by serum BD5 (F1: Sister 2) (Fig. 5.6*b*, lane 10). In contrast, sera that contained anti-U1 RNP antibodies, such as BD1 (F1: Patient) (Fig. 5.6*b*, lanes 6 and 11), or anti-PADPRP antibodies (Fig. 5.6*b*, lanes 8

and 9) were able to precipitate these antigens to the same degree regardless of which depleted extract was used.

When the study sera were subjected to IP studies using the BD5-antigen-depleted extract, most of the study sera were still able to precipitate 115-kDa antigens (Fig. 5.6*c*, lanes 2-6 and 9). However, as well as serum from BD5 (F1: Sister 2), serum from BD3 (F1: Sister 1) was also unable to precipitate a 115-kDa antigen from the BD5-antigen-depleted extract (Fig. 5.6*c*, lanes 8 and 7 respectively).

Similarly, serum from BD2 (F1: Husband) was used to specifically deplete a third batch of the same original extract (Fig. 5.6*d*, lanes 10 and 9). A control extract, depleted using a normal serum (Fig. 5.6*d*, lanes 2 and 1) was shown still to contain the 115-kDa antigen recognized by serum BD2 (F1: Husband) (Fig. 5.6*d*, lane 4). Again, sera that contained anti-U1 RNP or anti-PADPRP antibodies were able to precipitate these antigens from both depleted extracts (Fig. 5.6*d*, lanes 8 and 3, and 6 and 5, respectively). When the study sera were subjected to IP using the BD2-antigen-depleted extract, most of the study sera could still precipitate 115-kDa antigens (Fig. 5.6*e*, lanes 3-6, 8 and 9). However, as well as serum from BD2 (F1: Husband), serum from BD7 (F1: Sister 3) was also unable to precipitate a 115-kDa antigen from the BD2-antigen-depleted extract (Fig. 5.6*e*, lanes 7 and 10, respectively).

These findings indicate that serum from BD5 (F1: Sister 2) precipitated all antigens precipitated by serum BD3 (F1: Sister 1), and, also, that serum BD2 (F1: Husband) precipitated all antigens precipitated by serum BD7 (F1: Sister 3).

Immunoblotting of affinity purified autoantigens

Autoradiographs of nitrocellulose strips containing the 115-kDa antigen precipitated by serum from BD3 (F1: Sister 1) are shown in Fig. 5.7*a*. Figure 5.7*b* shows the same strips after blotting with the study sera. No serum recognized the 115-kDa band, even serum from BD3 itself (Fig. 5.7*b*, lane 4). When the nitrocellulose strips contained affinity purified 115-kDa antigen purified using serum from BD7 (Fig. 5.7*c*,), a similar result was obtained, with none of the study sera recognizing the 115-kDa band, even serum from BD7 itself (Fig. 5.7d, lane 6).

The 115-kDa antigens recognized by sera from patient LK1 (F4), and by sera from family members BD2 (F1: Husband), BD5 (F1: Sister 2), LJ7 (F3: Mother) and MD9 (F5: Son), were also successfully purified by the same method, as indicated by the presence of 115-kDa bands on autoradiographs (data not shown). In all these cases, when each set of IB strips was blotted using each of the test sera, no serum gave a positive result, even when the particular serum which had been used to purify the autoantigen concerned was used (data not shown).

It was thought possible that these negative results may have been due to low concentrations of antigens on the blotting strips. Therefore, similar purification procedures were carried out using two of the study sera (BD2 (F1: Husband) and BD5 (F1: Sister 2)), and increased quantities of extract and other reagents. The extract used

was not radiolabelled due to constraints on radioactive usage. However, as an alternative, it was intended to stain representative strips using the amido black technique in order to confirm successful purification of 115-kDa antigens, and their effective transfer to nitrocellulose sheets. Unfortunately, no success was experienced with this technique (data not shown): in retrospect, it may have been wiser to have transferred the purified proteins onto a more sturdy membrane such as PVDF, which can withstand Coomassie blue staining procedures. Nonetheless, the nitrocellulose blotting experiments were carried through with some success: although the 115-kDa antigen purified by serum from BD2 (F1: Husband) was not recognized by any of the study sera (Fig. 5.8*a*), including serum BD2 itself (Fig. 5.8*a*, lane 4), an antigen of ~115-kDa purified by serum from BD5 (F1: Sister 2) did now appear to be recognized by serum from BD5 as well as by serum from BD3 (F1: Sister 1) (Fig. 5.8*b*, lanes 2 and 5, respectively).

These results imply that the antigen recognized by serum from BD5 (F1: Sister 2) is recognized by antibodies contained in serum from BD3 (F1: Sister 1) and, further, that the autoantigenic epitopes recognized are likely to be linear (proteins are denatured during SDS-PAGE, however refolding of proteins on blots after electrophoretic transfer is possible).

Purification of autoantigens by immunoaffinity column chromatography

Study sera taken from BD2 (F1: Husband) and BD5 (F1: Sister 2) were used to successfully purify 115-kDa antigens, as shown by the presence of bands of the appropriate molecular weight in the silver-stained, eluted fractions of the mini-columns (Fig. 5.9*b* and *c*, respectively). The control mini-column, which contained Protein-A Sepharose beads covalently linked with antibodies from a normal serum, did not produce a band of 115-kDa when subjected to the same elution conditions (Fig. 5.9*a*).

The relevant fractions were further purified by de-salting, as described in Method 2.3.4*b*, and, following SDS-PAGE and transfer to PVDF membranes, Coomassie blue staining revealed the presence of a 115-kDa band in both cases (data not shown). However, when sent for sequencing, these samples were not of sufficient quantity to enable sequencing.

A more efficient antigen purification was achieved using the full-sized column, which contained Protein-A Sepharose bound to antibodies from serum BD3 (F1: Sister 1), as shown by the appearance of the silver-stained, eluted fractions in Fig. 5.9*d*. Once again, silver-stained elution fractions from the full-sized control column did not produce a band of 115-kDa (data not shown). The relevant fractions were further purified, as before, and, following SDS-PAGE and transfer to PVDF membranes, Coomassie-blue staining revealed the presence of a strong 115-kDa band (Fig. 5.10). This band was excised and sent for sequencing. However, although plenty of protein was found to be present, the protein was reported to be blocked at the NH_2-terminal, and was unsuitable for direct sequencing.

As described in Method 2.3.4*d*, CNBr may be used to cleave such proteins, allowing separation and sequencing of polypeptide fragments. Therefore, a further batch of the protein was prepared. This was subjected to a more thorough purification procedure (see Method 2.3.4*d*) in order to remove low molecular weight polypeptide contaminants. Following CNBr-induced fragmentation, the resulting polypeptides were SDS-PAGE separated and transferred to PVDF as before. However, to date, sufficient quantities of polypeptides to allow sequencing have not been isolated.

TABLE 5.1 Sera from SSc patients and SSc-free family members which precipitated unidentified autoantigens of 115-kDa in the SSc family study. (*a*) SSc patients; (*b*) family members of SSc patients; (*c*) family members of SSc patient BD1 (all members of this family are shown)

(*a*)

Sample	Sex	Clinical features	Unidentified bands by IP (kDa)	Defined auto-antibodies	Nuclear IF score	Nucleolar IF score
B1	F	lc-SSc	49,57,115,190	U3 RNP	-	++++cl./sp.
K1	F	lc-SSc	115 (wk)	-	++cs.sp.	-
LK1*	F	lc-SSc	115	Ro	+++dis.cs.sp.	-
NRM1	F	lc-SSc	115,125	topo I[†],Ro	++dif.f.sp.	+++h.

(*b*)

Sample	Sex	Clinical features	Relation to proband	Unidentified bands by IP (kDa)	Defined auto-antibodies	Nuclear IF score	Nucleolar IF score
BE6*	F	RP	Mother	115[§]	-	+/-gr.sp	+++sp.
LJ7*	F	-	Mother	115[§]	-	+f.sp.	-
MD9*	M	-	Son	115	-	+sp.	+++sp.

(*c*)

Sample	Sex	Clinical features	Relation to proband	Unidentified bands by IP (kDa)	Defined auto-antibodies	Nuclear IF score	Nucleolar IF score
BD1	F	dc-SSc	Proband	-	U1 RNP	++++cs.dis.sp.	-
BD2*	M	-	Husband	115	-	+f.sp.	+sp.
BD3*	F	RP	Sister	115	-	+++f.sp.	+/-
BD5*	F	RP	Sister	115	-	+++gr.sp.	+sp.
BD7*	F	-	Sister	115	-	-	-
BD4[¶]	F	-	Mother	-	-	-	-
BD6	M	-	Son	-	-	-	+sp./h.

*Sera selected for further study
[†]Also identified by immunodiffusion
[§]Unidentified precipitin also detected by immunodiffusion
[¶]This serum was not included in the SSc family study, but an 8-year-old sample was tested subsequently
IP, immunoprecipitation; IF, indirect immunofluorescence; lc-SSc, limited cutaneous SSc; dc-SSc, diffuse cutaneous SSc; RP, Raynaud's phenomenon; wk, weak; cs. course; cl. clumpy; sp. speckled; h. homogeneous; f. fine; dis. discrete; dif. diffuse; gr. grainy

TABLE 5.2 Details of sera precipitating unidentified 115-kDa autoantigens which were selected for further study. Revised indirect immunofluorescence (IF) results, carried out using a different brand of HEp-2 slides, are included (see text): *c.f.* Table 5.1

							Cytoplasmic IF score		Nuclear IF score		Nucleolar IF score	
Family number (F)	**Relation to proband**	**Sample identity**	**Sex**	**Age**	**Clinical features**	**Subjective intensity of IP band**	Pattern	Titre	Pattern	Titre	Pattern	Titre
F1:	Husband	BD2	M	77	-	++	+/-cs.sp.	1/640	-	-	-	-
	Sister 1	BD3	F	66	RP	++++	-	-	+++f.sp.	1/2560	-	-
	Sister 2	BD5	F	71	RP	++++	-	-	++++f.sp.	1/2560	-	-
	Sister 3	BD7	F	69	-	+++	+cs.sp.	1/640	-	-	-	-
F2:	Mother	BE6*	F	75	RP	+	++gr.fil.sp.	1/640	-	-	++sp.	1/640
F3:	Mother	LJ7*	F	75	-	++	++f.sp.	1/640	++f.sp.	1/640	+	1/40
F4:	Proband	LK1	F	46	lc-SSc	++	+/-f.sp.	1/40	++gr.sp.	1/640	++gr.sp.	1/640
F5:	Son	MD9	M	14	-	++	+/-f.sp.	1/40	+f.gr.sp.	1/40	++sp.	1/640

*Unidentified precipitin also detected by immunodiffusion

RP, Raynaud's phenomenon; lc-SSc, limited cutaneous SSc; IP, immunoprecipitation; wk. weak; cs. course, sp. speckled; f. fine; gr. grainy; dis. discrete; dif. diffuse; h. homogeneous; cl. clumpy; fil. filamentous

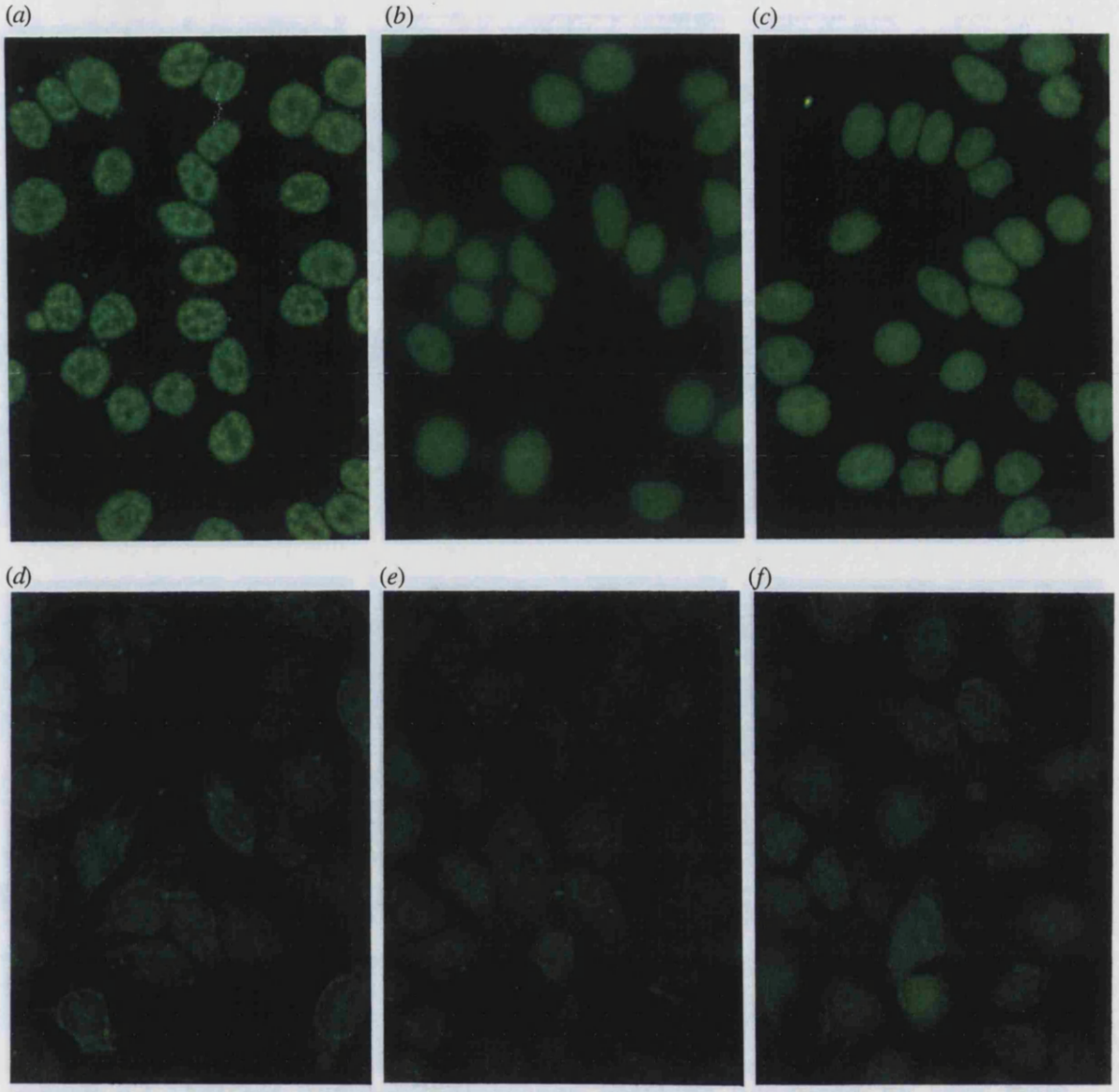


FIGURE 5.1 Indirect immunofluorescence patterns produced by sera from an SSc patient and from five of her connective tissue disease-free family members in the SSc family study. The original brand of HEp-2-cell slides was used (see text). (*a*) Serum from SSc proband BD1 contained anti-U1 RNP antibodies, and produced a very strong course speckled nuclear stain, nucleolar sparing; cytoplasmic staining was not present. (*b*) Serum from BD3, a Raynaud's phenomenon (RP)-affected sister of BD1, produced a strong diffuse fine speckled nuclear pattern, with very weak speckled nucleolar staining: cytoplasmic staining was not present. Serological tests had failed to identify antibodies of defined specificity: however, a very strong, unidentified 115-kDa band was apparent by immunoprecipitation (IP). (*c*) Serum from another RP-affected sister, BD5, produced a diffuse fine/grainy speckled nuclear pattern, with a weak speckled nucleolar pattern; cytoplasmic staining was not present. Although no antibodies of defined specificity had been detected by serological tests, a very strong, unidentified 115-kDa band was apparent by IP. (*d*) Serum from BD7, the healthy sister of BD1, produced a weak grainy/filamentous cytoplasmic pattern, while nuclear and nucleolar immunofluorescence (IF) were scored as negative. Serological tests had failed to identify antibodies of defined specificity: however, a strong, unidentified 115-kDa band was apparent by IP. (*e*) Serum from BD6, the healthy son of BD1 was scored as '+' for nucleolar IF only. Serological tests failed to detect defined autoantibody specificities, and no unidentified bands were apparent by IP. (*f*) A weak diffuse fine speckled nuclear pattern, with weak staining of the nucleolus, and a very weak grainy cytoplasmic pattern, was produced by serum from BD2, the healthy husband of BD1. Although no antibodies of defined specificity had been detected by serological tests, a strong, unidentified 115-kDa band was apparent by IP.

FIGURE 5.2 Indirect immunofluorescence patterns produced in the present study by sera precipitating a 115-kDa band by radioimmunoprecipitation. A different brand of HEp-2-cell slides was used (see text). The staining patterns produced by the study sera were found to be more distinctive than they had been with the original slides (see Table 5.2, *c.f.* Table 5.1 and Fig. 5.1). A variety of other sera are included for comparison, each of which had also been tested using the original study slides, and some of these, too, produced slightly different staining patterns. (*a*) serum from BD1, the U1 RNP-positive proband from Family 1, produced a staining pattern similar to that observed previously (*c.f.* Fig. 5.1*a*). (*b*) serum from BD2, the husband of BD1, produced a weak, but more definite, cytoplasmic staining pattern, with no nuclear or nucleolar staining (*c.f.* Fig. 5.1*f*). (*c*) serum from BE6, the Raynaud's phenomenon-affected mother of proband BE1, produced a much clearer cytoplasmic staining pattern, while nucleolar staining was slightly weaker than before (*c.f.* previous Fig. 3.8*f*). (*d*) serum from proband LK1 also produced weaker staining than before. (*e*) The pattern produced by this normal control serum was shown previously in Fig. 3.1*a*, where it was scored as negative; similarly, (*f*) anti-PL-7 prototype (*c.f.* previous Fig 3.2*c*) and (*g*) anti-Ro/La serum (*c.f.* previous Fig. 3.3*c*) produced slightly stronger patterns than they had previously. (*h*) serum from BA2, the healthy sister of proband BA1 produced more prominent nucleolar staining than before (*c.f.* previous Fig. 3.8*h*), with no cytoplasmic staining. *(Continued overleaf...)*

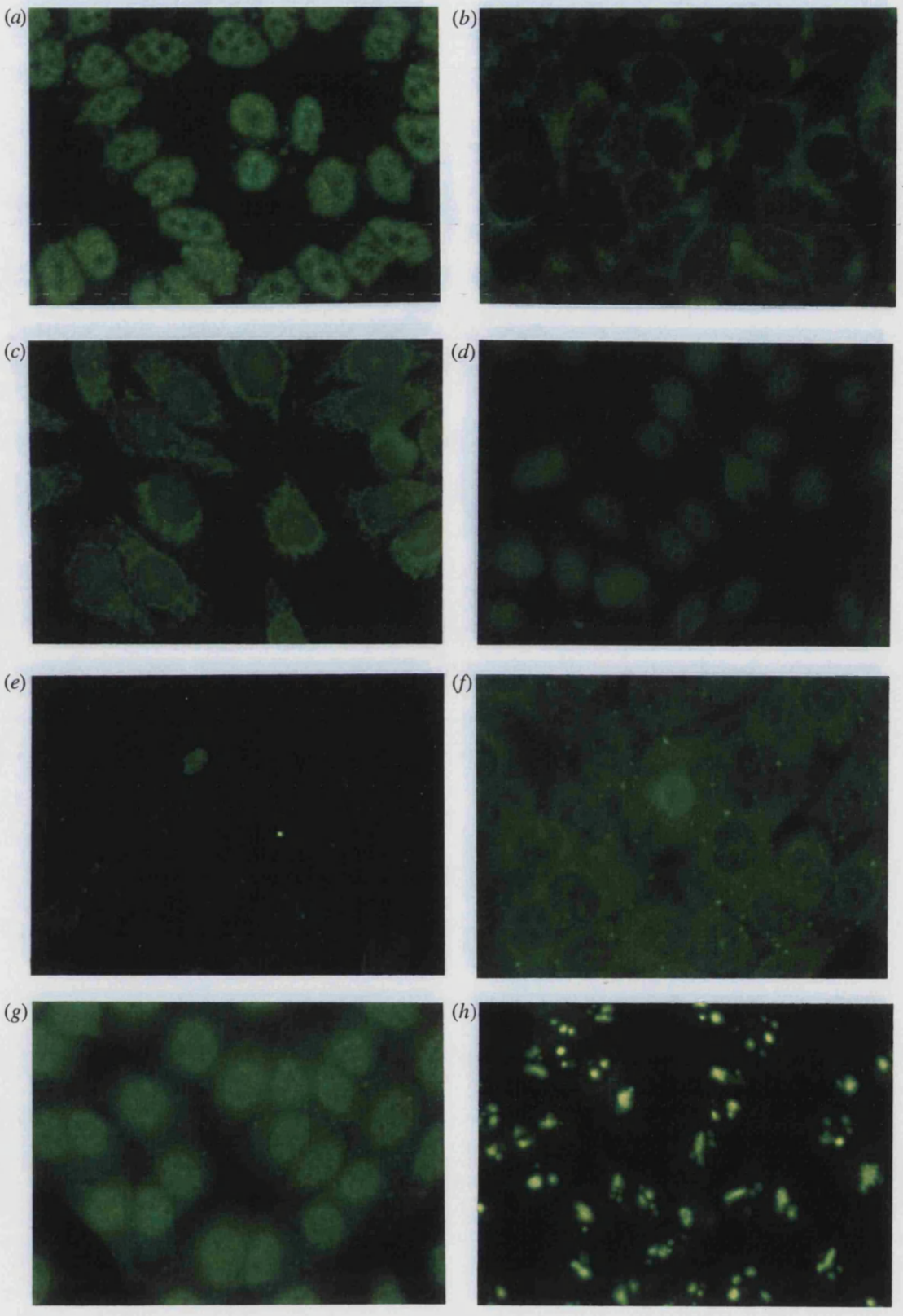


FIGURE 5.2 *(cont.)* Indirect immunofluorescence patterns produced in the present study by sera precipitating a 115-kDa band by radioimmunoprecipitation

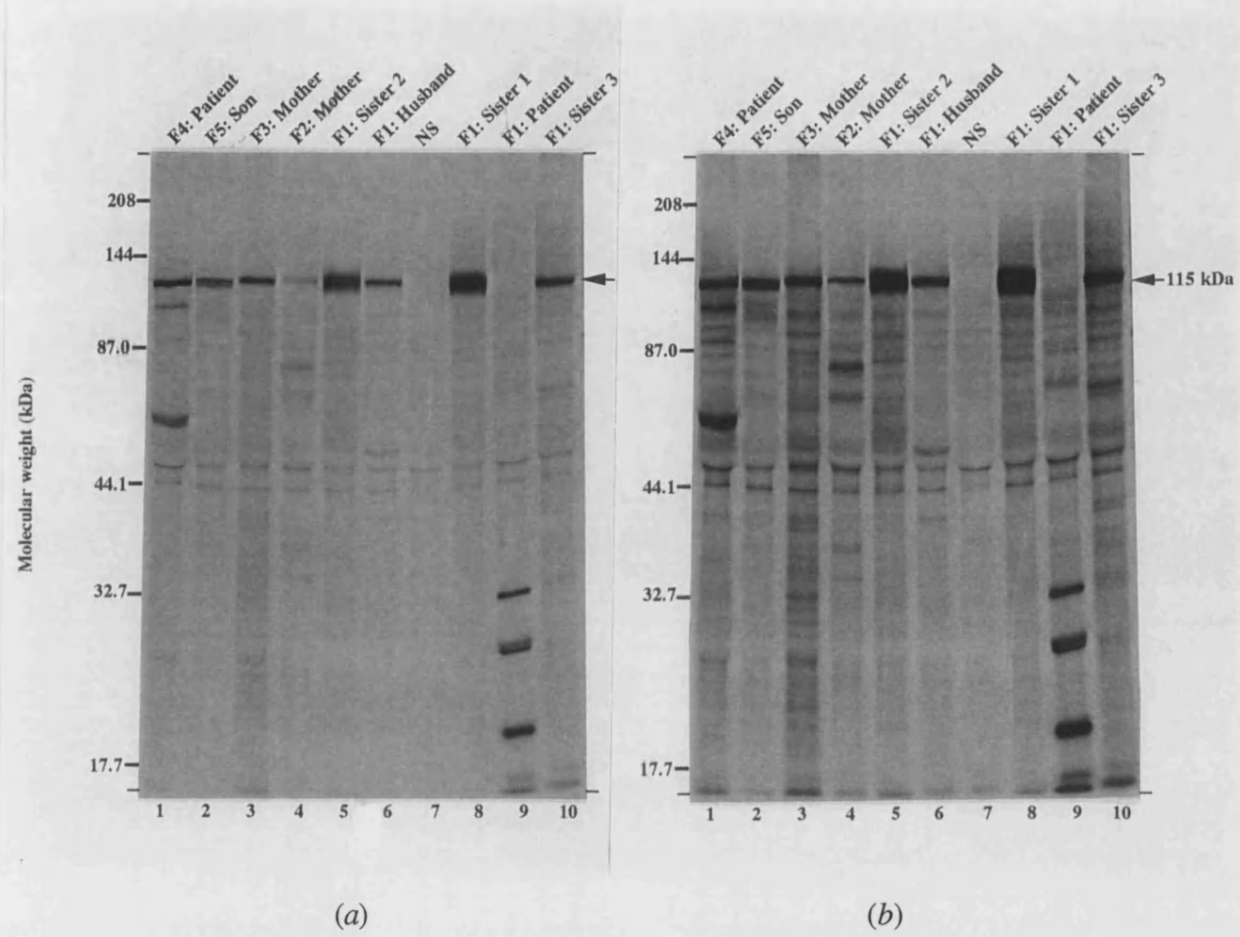
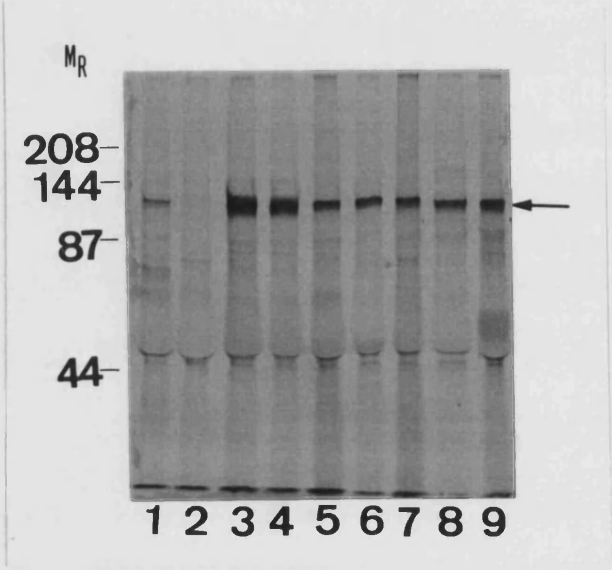


FIGURE 5.3 Radioimmunoprecipitation: relative band intensities of unidentified 115-kDa autoantigens precipitated by the study sera. Autoradiographs of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. (*a*) Lane 7, normal serum; lane 9, proteins of the U1 RNP complex precipitated by SSc patient BD1 from Family 1 (F1: Patient). Serum from SSc patient LK1 (F4: Patient) precipitated an unidentified 115-kDa protein as well as the Ro autoantigen (lane 1); serum from SSc-free family members MD9 (F5: Son), LJ7 (F3: Mother), BE6 (F2: Mother), BD5 (F1: Sister 2), BD2 (F1: Husband), BD3 (F1: Sister 1) and BD7 (F1: Sister 3) precipitated 115-kDa proteins (lanes 2-6, 8 and 10 respectively). (*b*) A longer exposure of the same gel was compared with (*a*): subjective relative band intensities were as follows: BD5 and BD3, very strong; BD7, strong; LK1, MD9, LJ7, and BD2, moderate; BE6, weak (see Table 5.2).



Key:

1. BE6 (F2: Mother)
2. Normal serum
3. BD5 (F1: Sister 2)
4. BD3 (F1: Sister 1)
5. BD7 (F1: Sister 3)
6. BD2 (F1: Husband)
7. LJ7 (F3: Mother)
8. MD9 (F5: Son)
9. LK1 (F4: Proband)

FIGURE 5.4 Radioimmunoprecipitation: relative electrophoretic mobilities of unidentified ~115-kDa autoantigens precipitated by the study sera. Autoradiographs of SDS-PAGE-separated ^{35}S-methionine-labelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. Samples were run on a 7.5%-polyacrylamide gel: antigens precipitated by all study sera (BE6, BD5, BD3, BD7, BD2, LJ7, MD9, and LK1) appeared to have very similar electrophoretic mobilities, and, therefore, molecular weights (lanes 1 and 3-9 respectively). Lane 2, normal serum.

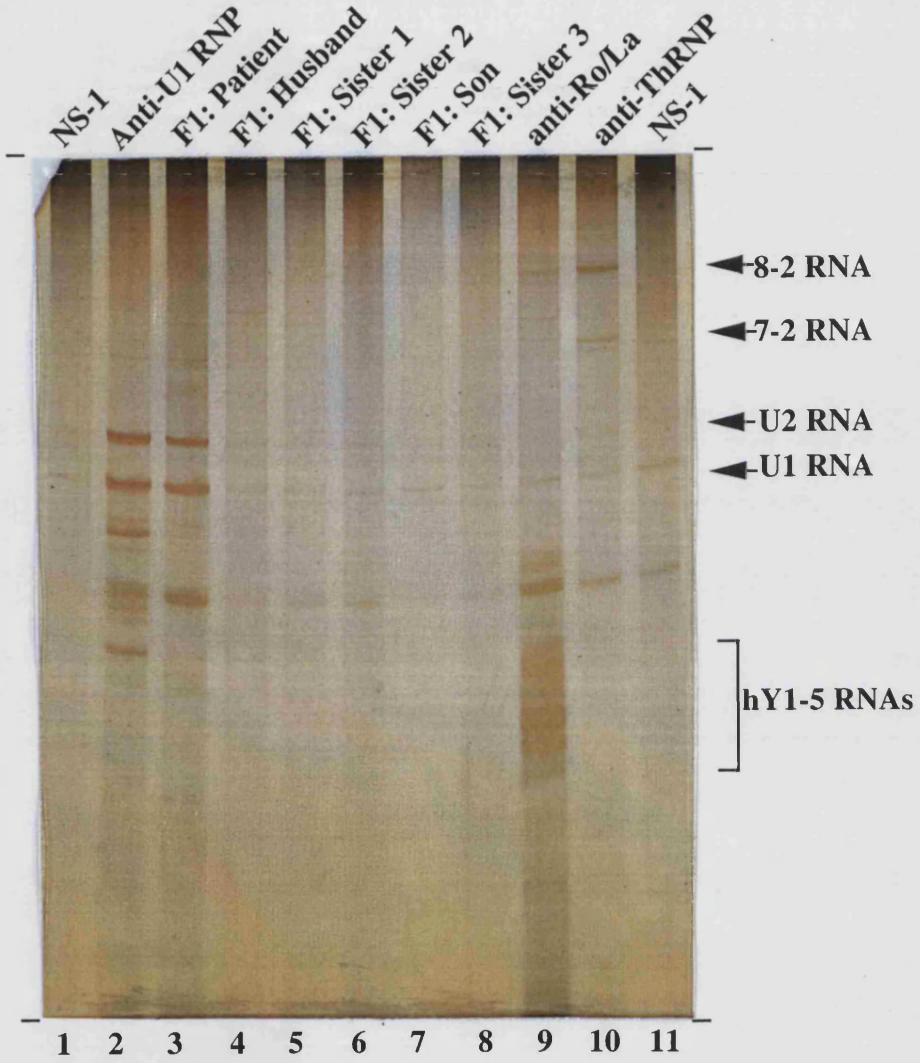
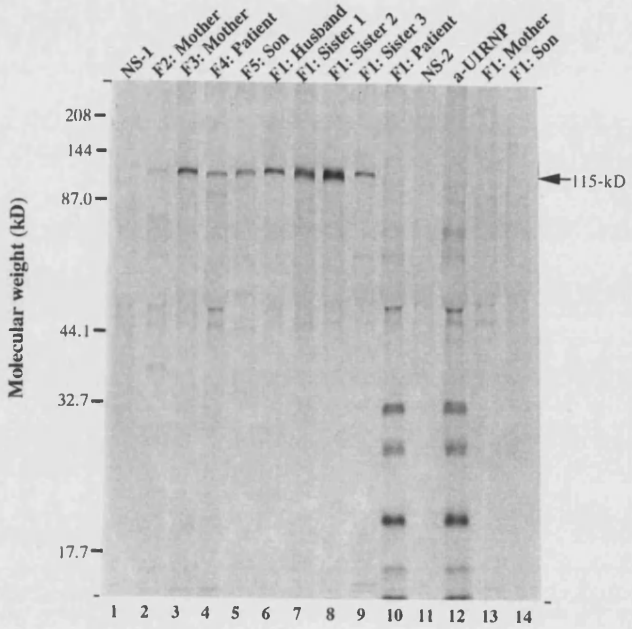


FIGURE 5.5 RNP-immunoprecipitation: RNA analysis of unidentified 115-kDa proteins precipitated by study sera from a single family. Silver-salt staining of SDS-PAGE-separated unlabelled proteins immunoprecipitated from K562-cell extracts by serum antibodies attached to Protein-A Sepharose beads. As indicated by arrows, the prototype anti-U1 RNP serum in lane 2 precipitated both U1 and U2 RNAs; prototype Ro/La serum in lane 9 precipitated the hY1-5 RNAs, while the anti-Th RNP serum in lane 10 precipitated the 7-2 and 8-2 RNAs, as expected. Serum from SSc patient BD1 (Family 1 (F1): Patient; lane 3) also precipitated U1 and U2 RNAs. However, no RNAs were precipitated by sera from the SSc-free relatives (BD2 (F1: Husband), BD3 (F1: Sister 1), BD5 (F1: Sister 2), BD6 (F1: Son), BD7 (F1: Sister 3)) or BD1 (F1: Patient) (lanes 4-8 respectively)): *c.f.* lanes 1 and 11, normal sera.



(*a*)

FIGURE 5.6 Radioimmunoprecipitation of unidentified 115-kDa autoantigens by sera from connective tissue disease (CTD)-free family members using a K562-cell extract precleared of certain 115-kDa autoantigens. A batch of radiolabelled K562-cell extract was specially prepared, and divided into three lots. (*a*) Test sera previously found to precipitate an unidentified 115-kDa band were then included in IP assays using the first lot of whole extract (lanes 2-9). Other members of the BD family (Family 1 (F1)) are included for comparison: patient BD1 (F1: Patient, lane 10) had only anti-U1RNP antibodies, as shown by comparison with an anti-U1 RNP prototype serum (lane 12), while serum from the patient's mother BD4 (F1: Mother, lane 13), and the patient's son BD6 (F1: Son, lane 14) contained no detectable autoantibodies. Lanes 1 and 11, normal sera. *(Continued overleaf...)*

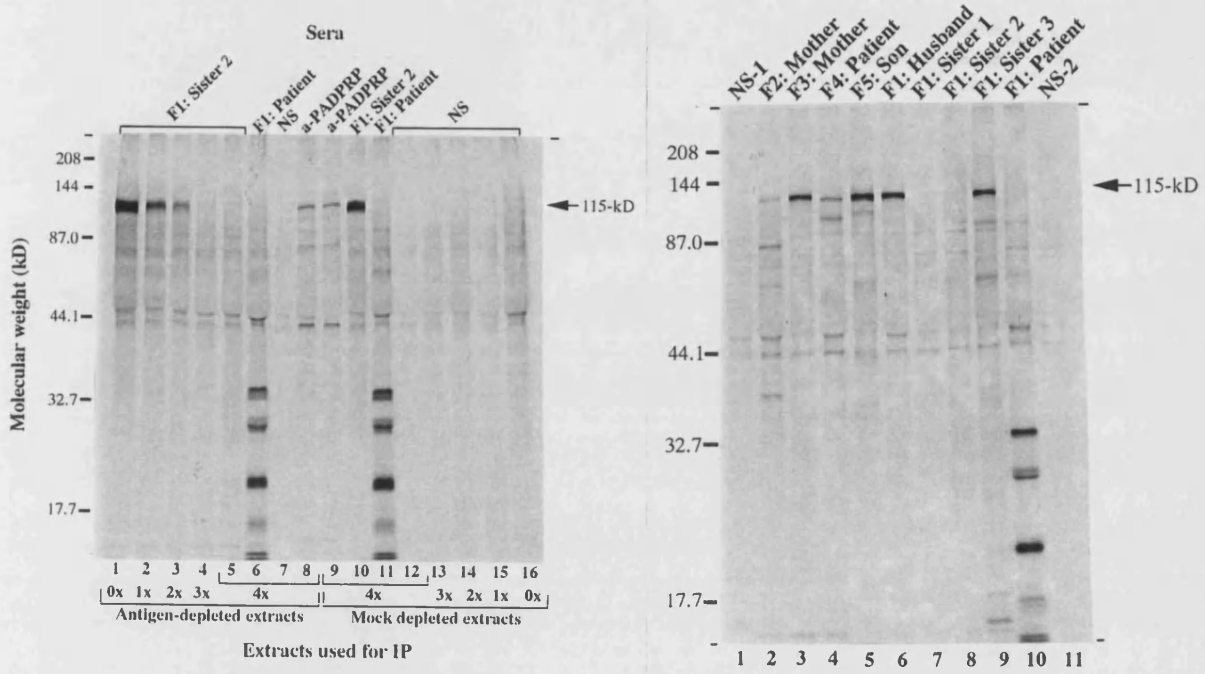


FIGURE 5.6 Radioimmunoprecipitation of unidentified 115-kDa autoantigens by sera from CTD-free family members using a K562-cell extract precleared of certain 115-kDa autoantigens. *(cont.)* (*b*) A second lot of whole extract was depleted of an unidentified 115-kDa autoantigen four times using Protein A-Sepharose beads preincubated with serum from BD5 (F1: Sister 2). The 115-kDa band is present in the whole extract (lane 1), and reduced by successive depletions (lanes 2-5), being absent from the 4x-depleted extract (lane 5). However, other antigens, such as U1 RNP and poly(ADP-ribose) polymerase (PADPRP) could still precipitated by appropriate sera (lanes 6 and 8 respectively). A normal serum is shown for comparison in lane 7. A control sample of whole extract was "depleted" in the same way using NS (lanes 12-16). This extract was shown to contain U1 RNP (lane 11), PADPRP (lane 9) as well as the unidentified 115-kDa antigen recognized by serum BD5 (F1: Sister 2; lane 10). (*c*) Sample sera which precipitated a 115-kDa band from the whole extract (*a*) were then included in IP assays using the fraction of extract depleted by serum BD5 (F1: Sister 2) (lanes 2-9). A 115-kDa band was no longer precipitated by serum BD3 (F1: Sister 1; lane 7). However, all other anti-115-kDa sera still precipitated a 115-kDa band (lanes 2-6 and 9). The patient BD1 (F1: Patient) still precipitated the U1 RNP complex (lane10). *c.f.* lanes 1 and 11, normal human sera. *(Continued overleaf...)*

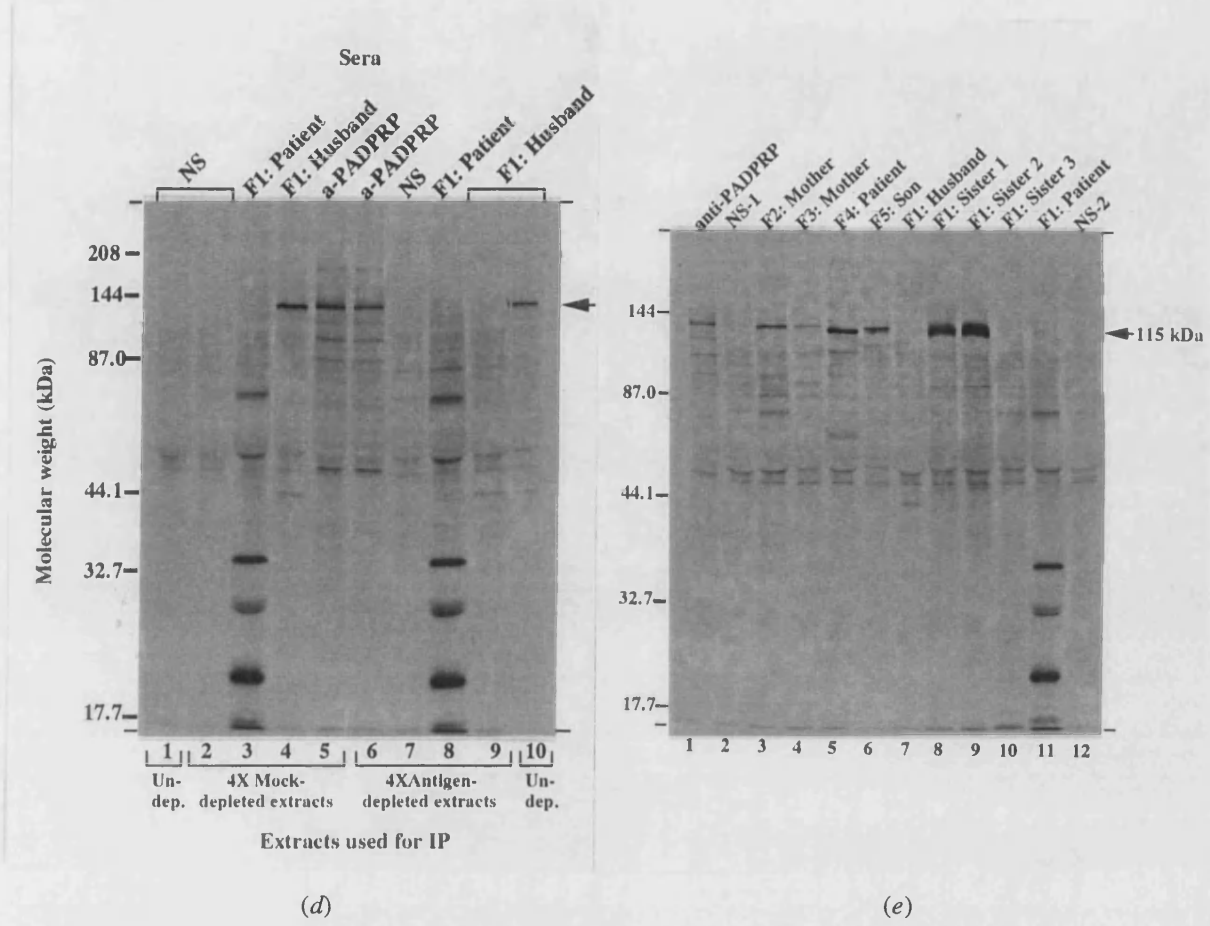
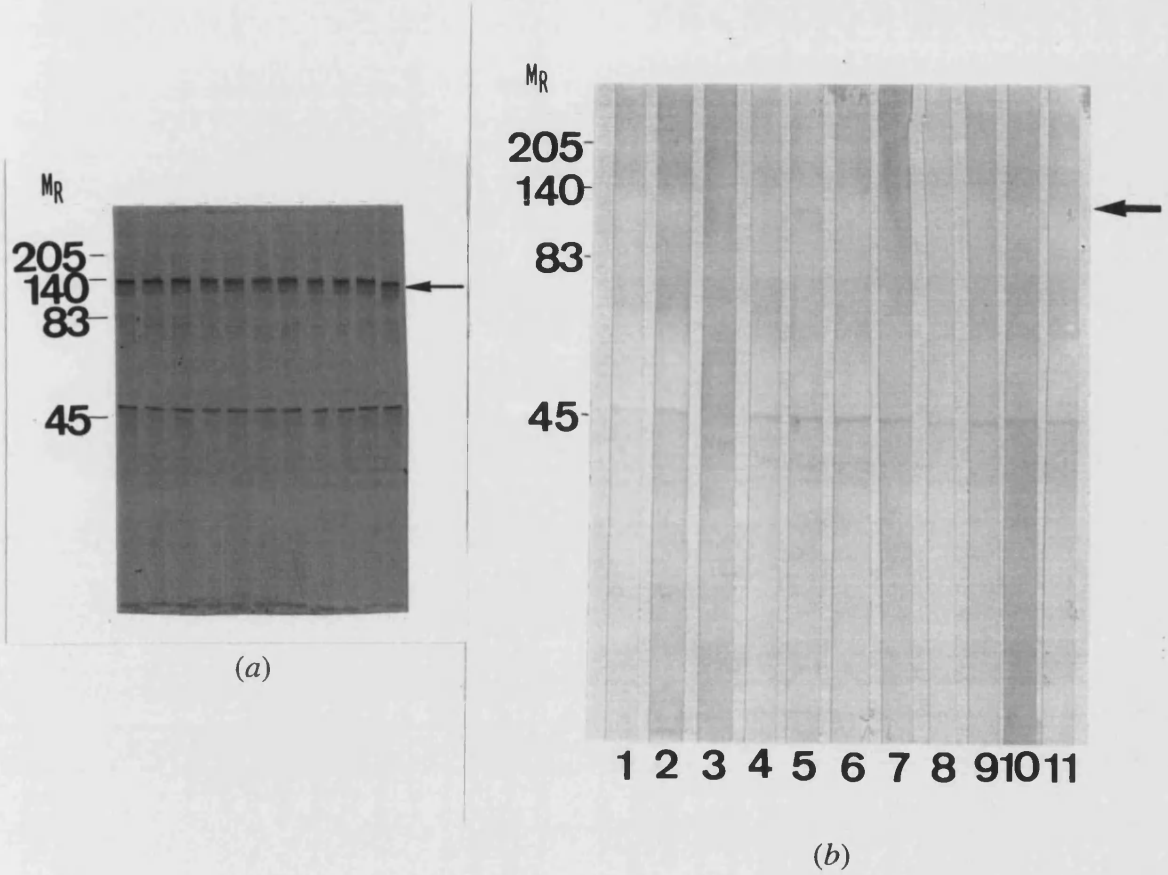


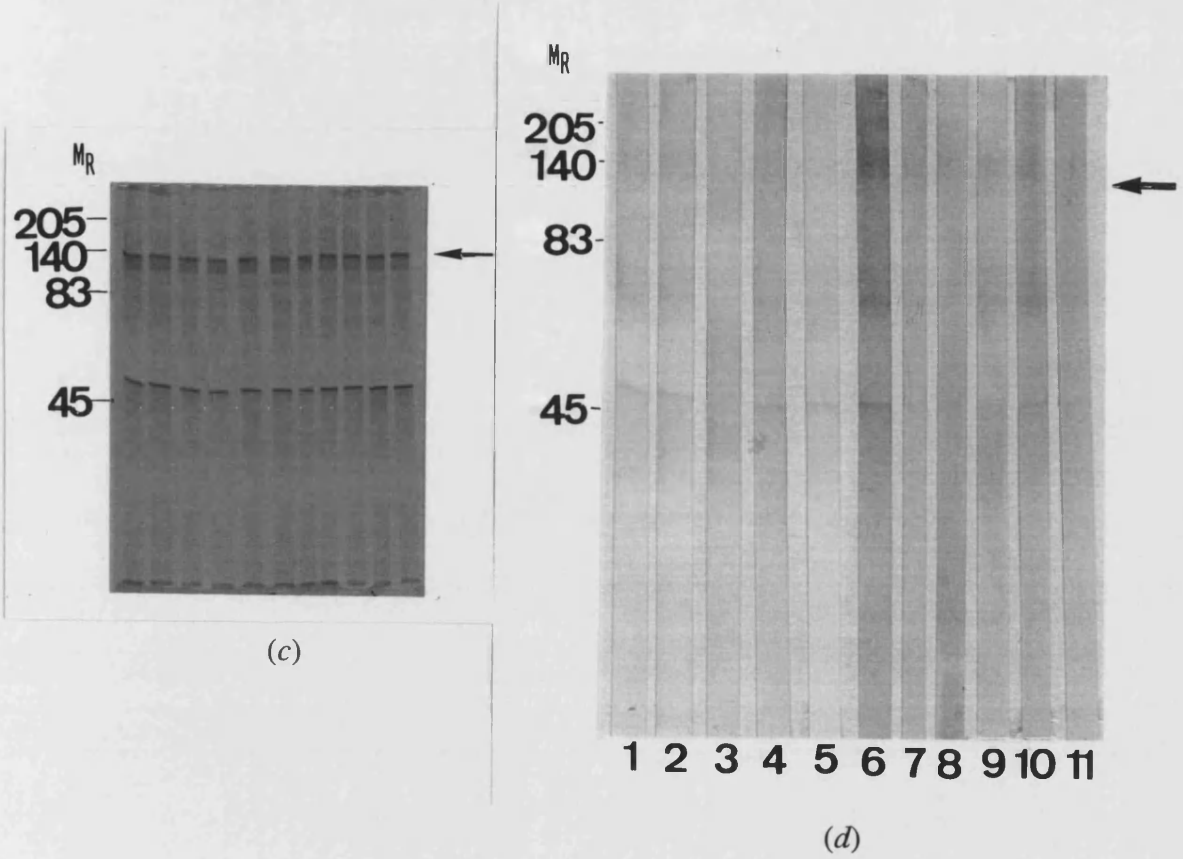
FIGURE 5.6 Radioimmunoprecipitation of unidentified 115-kDa autoantigens by sera from a CTD-free family member using a K562-cell extract precleared of certain 115-kDa autoantigens. ***(cont.)*** (*d*) A third lot of whole extract was depleted of an unidentified 115-kDa autoantigen four times using Protein A-Sepharose beads preincubated with serum BD2 (F1: Husband). The 115-kDa band is present in the whole extract (lane 10), and reduced by successive depletions, being absent from the 4x-depleted extract (lane 9). However, U1 RNP and PADPRP could still be precipitated by appropriate sera (lanes 8 and 6 respectively). A normal serum is shown for comparison in lane 7. A control sample of whole extract was "depleted" in the same way using NS (lanes 1 and 2), and was shown to contain U1 RNP (lane 3), PADPRP (lane 5), as well as the unidentified 115-kDa antigen recognized by serum BD2 (F1: Husband; lane 4). (*e*) Sample sera which precipitated a 115-kDa band from the whole extract (*a*) were then included in IP assays using the fraction of extract depleted by serum BD2 (F1: Husband) (lanes 3-10). A 115-kDa band was no longer precipitated by sera from BD2 (F1: Husband) or BD7 (F1: Sister 3) (lanes 7 and 10 respectively). However, all other anti-115-kDa sera still precipitated a 115-kDa band (lanes 3-6, 8 and 9). *c.f.* lanes 2 and 12, normal human sera.



Key:

1	NS	6	BD7 (F1: Sister 3)
2	BD1 (F1: Patient)	7	BD6 (F1: Son)
3	BD2 (F1: Husband)	8	LJ7 (F3: Mother)
4	BD3 (F1: Sister 1)	9	MD9 (F5: Son)
5	BD5 (F1: Sister 2)	10	LK1 (F4: Patient)
		11	anti-topo/Ro/La standard

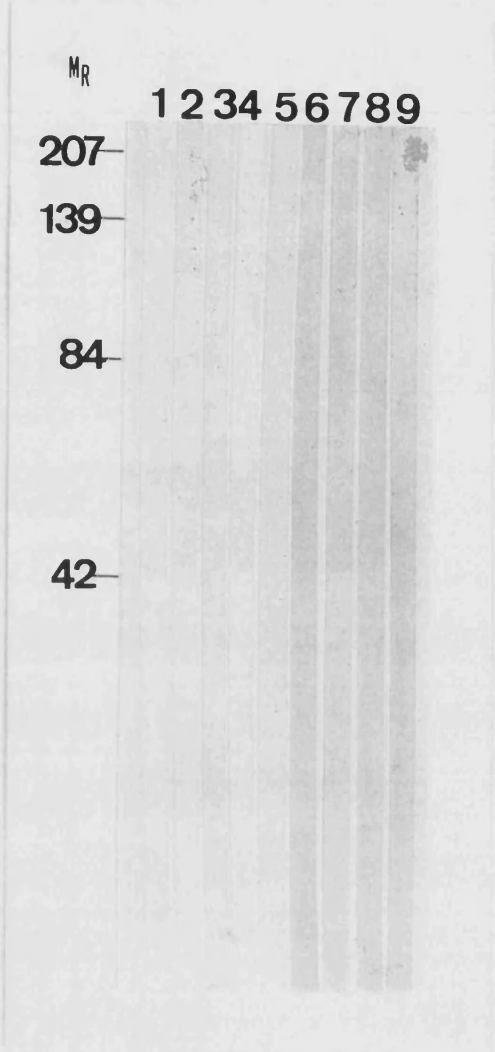
FIGURE 5.7 Detection of affinity purified, radiolabelled 115-kDa autoantigens by immunoblotting. (*a*) Antigens were affinity purified by radioimmunoprecipitation using serum BD3 (Family 1 (F1): Sister 1) which had previously been found to precipitate an unidentified protein with a molecular weight of 115-kDa (*b*) Following SDS-PAGE of the autoantigen, and its transfer to nitrocellulose, immunoblotting was carried out using the test sera. Subsequent autoradiography of blots revealed the presence of the 115-kDa protein (*a*) (arrowed). However, none of the study sera (BD2, BD3, BD5, BD7, LJ7, MD9 and LK1) contained antibodies which recognized the band, including serum BD3 itself (*b*, lanes 3-6 and 8-10 respectively). Lane 1, normal serum (NS); lane 2, patient BD1 (anti-U1 RNP); lane 7, BD6; lane 11, anti-topo I/Ro/La standard. *(Continued. overleaf..)*



Key:

1	NS	6	BD7 (F1:Sister 3)
2	BD1 (F1: Patient)	7	BD6 (F1: Son)
3	BD2 (F1: Husband)	8	LJ7 (F3: Mother)
4	BD3 (F1: Sister 1)	9	MD9 (F5: Son)
5	BD5 (F1: Sister 2)	10	LK1 (F4:Patient)
		11	anti-topo/Ro/La standard

FIGURE 5.7 Detection of affinity purified, radiolabelled 115-kDa autoantigens by immunoblotting. *(cont.)* (*c*) Antigens were affinity purified by radioimmunoprecipitation using serum BD7 (F1: Sister 3), which had previously been found to precipitate an unidentified protein with a molecular weight of 115-kDa (*d*) Following SDS-PAGE of the autoantigen, and its transfer to nitrocellulose, immunoblotting was carried out using the test sera. Subsequent autoradiography of blots revealed the presence of the 115-kDa protein (*c*) (arrowed). However, none of the study sera (BD2, BD3, BD5, BD7, LJ7, MD9 and LK1) contained antibodies which recognized the band, including serum BD7 itself (*d*, lanes 3-6 and 8-10 respectively). Lane 1, normal serum (NS); lane 2, patient BD1 (anti-U1 RNP); lane 7, BD6; lane 11, anti-topo I/Ro/La standard.

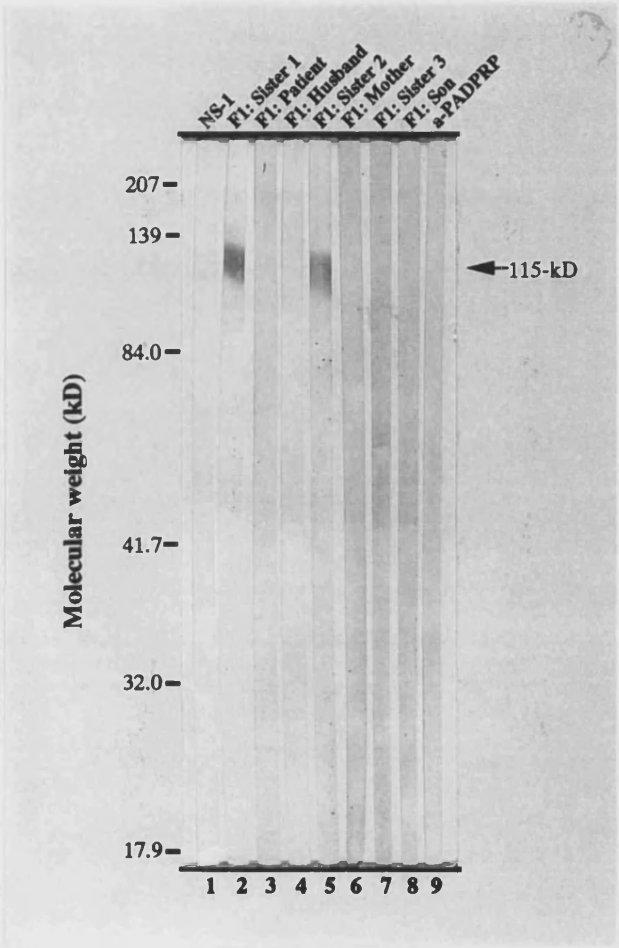


(*a*)

Key:

1 Normal serum	5 BD5 (F1: Sister 2)
2 BD3 (F1: Sister 1)	6 BD4 (F1: Mother)
3 BD1 (F1: Patient)	7 BD7 (F1: Sister 3)
4 BD2 (F1: Husband)	8 BD6 (F1: Son)
	9 anti-PADPRP mAb

FIGURE 5.8 Detection of unlabelled affinity purified 115-kDa autoantigens by immunoblotting of antigen-rich strips. Four SSc-free members of a single SSc family (BD2, BD3, BD5 and BD7) had been found to precipitate a 115-kDa band from K562-cell extracts. Unidentified 115-kDa autoantigens were affinity purified by immunoprecipitation using serum from BD2 and serum BD5 (*a* and *b*, respectively) Following SDS-PAGE of the autoantigens, and transfer to nitrocellulose, immunoblotting was carried out using sera from other members of this family (Family 1 (F1)). (*a*) Sera from BD3 (F1: Sister 1), BD5 (F1: Sister 2) and BD7 (F1: Sister 3) all failed to recognize the 115-kDa autoantigen precipitated by serum BD2 (F1: Husband) (lanes 2, 5 and 7 respectively). The band was not recognized by sera from other members of the BD family either, including serum BD2 itself (lanes 2, 3 and 6-8), or by the anti-poly(ADP-ribose) polymerase mAb (PADPRP; lane 9). *(Continued overleaf...)*



(*b*)

FIGURE 5.8 Detection of unlabelled affinity purified 115-kDa autoantigens by immunoblotting of antigen-rich strips. ***(cont.)*** (*b*) Serum from BD3 (F1: Sister 1) and serum from BD5 (F1: Sister 2) both recognized the 115-kDa antigen precipitated by serum BD3 (F1: Sister 1) (*a*, lanes 2 and 5 respectively). However, serum from BD2 (F1: Husband) and serum from BD7 (F1: Sister 3), did not recognize the antigen (*a*, lanes 4 and 7 respectively). Further, the band was not recognized by sera from the patient BD1 (F1: Patient; lane 3), the patient's mother BD4 (F1: Mother; lane 6) or the patient's son BD6 (F1: Son; lane 8). A normal serum (NS-1) is shown for comparison in lane 1.

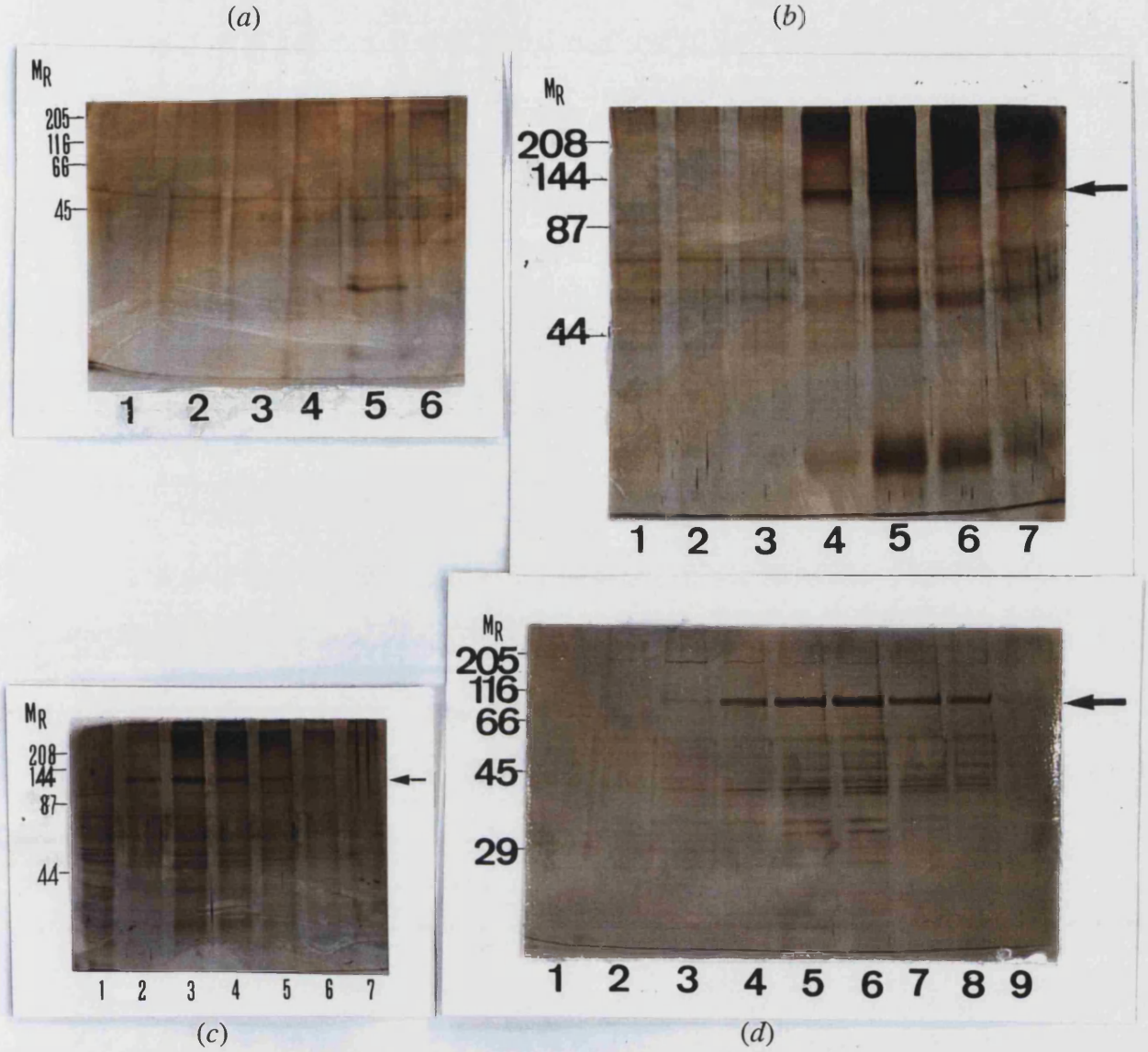


FIGURE 5.9 Protein silver staining: detection of purified 115-kDa autoantigens recognized by study sera, and eluted from an immunoaffinity column by a low pH, glycine buffer. During preliminary experiments, columns containing serum antibodies covalently attached to a 2-ml bed volume of Protein-A Sepharose were exposed to K562-cell extracts, washed, and eluted. Twenty 600-μl fractions of eluate were collected into 60 μl of Tris.Cl buffer (IM, pH9.0). A 20-μl sample of each fraction was then analyzed by SDS-PAGE on a 10%-polyacrylamide gel, followed by silver staining. (*a*) A precolumn containing normal serum-coated Protein-A Sepharose was used to preclear all extracts of non-specifically adsorbed proteins. This column was eluted before each re-use, and no bands of 115-kDa were detected in the eluted fractions (fractions shown in order of elution, lanes 1-7), although non-specifically binding proteins of lower molecular weight were detected (lane 5). (*b*) When this precleared extract was applied to a column containing Protein-A Sepharose beads linked to antibodies from serum BD2 (Family 1 (F1): Husband), however, a prominent band of 115-kDa was detected in eluted fractions 4-7 (lanes 4-7). Similarly, when precleared extracts were applied to a column containing Protein-A Sepharose linked to antibodies from BD5 (F1: Sister 2), a 115-kDa band was detected in eluted fractions (*c*, lanes 3-8). When fully scaled-up columns (5-ml bed volume) were later prepared using NS, and serum from BD3 (F1: Sister 1), a much cleaner preparation of antigen was eluted (1.2 ml fractions of eluate; 300 μl collection buffer) (*d*, lanes 2-6): fractions from lanes 2-4 were consequently selected for further purification.

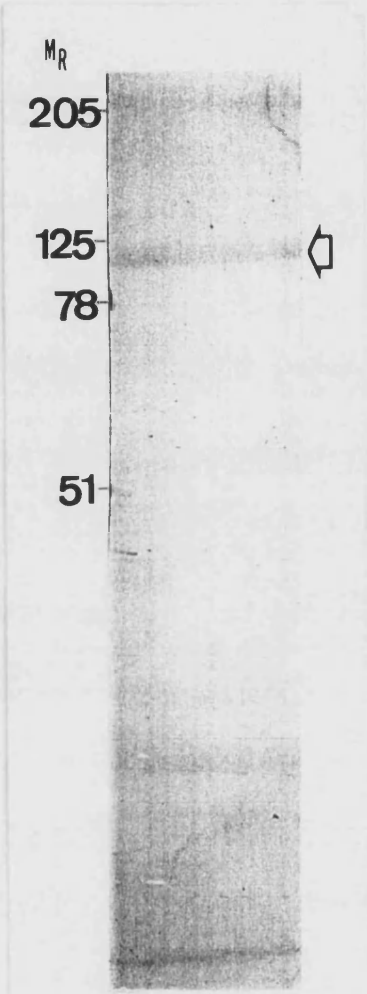


FIGURE 5.10 Coomassie blue staining: detection of a purified 115-kDa autoantigen recognized by one of the study sera and eluted from an immunoaffinity column by a low pH, glycine buffer. Antibodies contained in serum from family member BD3 (F1: Sister 1) were covalently bound to Protein-A Sepharose beads in an immunoaffinity column. After application of an extract of K562 cells, followed by elution with a low-pH glycine buffer, antigen-rich fractions (see Fig. 5.9) were further purified by desalting, and separated on a 10% SDS-polyacrylamide gel. After electrophoretic transfer to a PVDF membrane, the purified protein was visualized by Coomassie blue staining (arrowed). The band was then excised, and sent for amino-acid sequencing.

5.4 DISCUSSION

Indirect immunofluorescent localization studies

The images produced by the second brand of HEp-2-cell slides were more distinctive than those produced by the original slides, and it is possible that these clearer images were due to more effective fixing techniques. However, it should not be assumed that the antigen distribution patterns of the cells on these particular slides were necessarily more representative of the in-vivo reality of subcellular antigen localization.

Regardless of which brand of slides produced the most accurate representation, the differences observed in staining patterns and intensities when the same serum was tested on the two types of slide indicate that results obtained from IF studies are extremely variable, and that a major source of variability lies in the choice of HEp-2 slide used. Different fixing conditions may cause subtle changes to antigens or differential permeabilities of subcellular membranes to antigens and/or antibodies. Alternatively, the physiological state of the majority of cells prior to the fixing procedure may be a factor. Thus, the antigen profile of the cell substrate as well as the autoantibody profile of the test serum is a factor for consideration in IF studies. For effective comparisons, therefore, all samples should be prepared using slides from the same manufacturing batch, and all incubations should ideally be performed on the same day.

In the SSc family study, not only was the same brand of HEp-2 slides used within each separate experiment, but, in addition, slides from the same manufacturing batch were used. Thus, it is believed that the results presented in Chapter 3 are an accurate reflection of relative IF intensities within that particular system.

Possible identity of the autoantigens recognized by the study sera

To test the hypothesis that sera from this family were precipitating the 116-kDa enzyme PADPRP, an anti-PADPRP MAb was included as a test serum in the antigen depletion experiments. Despite specifically depleting extracts of the two different 115-kDa antigens recognized by sera from BD2 (F1: Husband) and BD5 (F1: Sister 2), it appeared that PADPRP could still be precipitated from both depleted extracts by the anti-PADPRP MAb, implying that none of the sera from this particular family contained antibodies which recognized PADPRP. Furthermore, the IF patterns of the BD family did not resemble those previously reported for PADPRP: using a HEp-2-cell substrate, Yamanaka *et al.* reported that autoimmune sera containing anti-PADPRP antibodies produce a distinctive diffuse nuclear fluorescence pattern, with intense nucleolar staining (369).

Information provided by the RNA-IP experiments showed that, apart from the proband BD1 (F1: Patient), no members of the BD Family precipitated an RNP.

Importantly, autoantibodies contained in serum BD1 did not precipitate a 115-kDa antigen, and the preclearing experiments did not affect the precipitation of any of the

subunits of the U1 RNP particle by BD1. Thus, different proteins were precipitated by BD1, and by the other members of her family. All other results reported here are in accordance with these findings, i.e. that the U1 RNP particle and each of the unidentified 115-kDa antigens were recognized by separate autoantibody systems.

Relationships between the different 115-kDa antigens

Although the various study sera were shown to precipitate at least three different 115-kDa proteins, the very close mobilities of all the 115-kDa antigens means that a fundamental relationship between the antigens is not ruled out, particularly in the case of the two different antigens which were each recognized by two members of the same family.

One possibility is that the antigen recognized by serum from BD3 (F1: Sister 1) and BD5 (F1: Sister 2) is a phosphorylated variant of that recognized by sera from BD2 (F1: Husband) and BD7 (F1: Sister 3). In support of this conjecture is the diffuse band produced on IP gels by the first two sera: such bands have been associated with phosphorylated proteins, such as the phosphorylated IIo subunit of RNAP II. As clearly illustrated in Chapter 4, two alternatively phosphorylated forms of a protein can be recognized by discrete sets of sera. Furthermore, cells have been shown to use phosphorylation as a localization signal to direct the segregation of a particular enzyme between different subcellular compartments, this being a form of controlling activity. In this regard, it may be significant that the two antigens mentioned above were located in separate cellular compartments, as indicated by the present IF results.

5.5 CONCLUSIONS

The involvement of an environmental trigger is suggested

As Kahaleh has pointed out (167), a single case report may contribute to the elucidation of pathogenetic mechanisms more than in-depth, wide-ranging laboratory studies. Similarly, the results concerning the autoantibody profile of members of the BD family provoke thoughts on aetiology and pathogenesis in SSc, despite the fact that only a single kindred has been described.

The spouse of an SSc-affected individual has been shown to have developed an autoimmune response, but not the disease itself. The apparent occurrence of the same autoantibody system in one of the patient's sisters implies an environmental factor is involved in the production of this particular autoantibody, and it is tempting to speculate that the same environmental factor may be responsible for triggering the disease in the proband, even though the patient recognized a different autoantigen. Furthermore, an autoantigen of the same molecular weight was recognized by the other two sisters in this family. In short, the evidence presented here is strongly in favour of an environmental factor triggering disease in the proband, while, in the remaining

family members, the same proposed environmental agent provoked one of a number of alternative responses.

A connection between the pathogenesis of RP and SSc is supported

This variable response of the different members of a single family to the same environmental stimulus could have depended on individual genetic susceptibility factors. As mentioned in a recent review by Rosen *et al.*, susceptibility factors contributing to the development of SSc are believed to include a pre-existing vascular defect (288,164,167). In this respect, it was of particular interest that BD3 and BD5, whose sera appeared to recognize the same protein, both had RP, but did not have SSc. Furthermore, it has been shown that there is a tendency for RP to cluster in certain families (100). Thus, along with the proband, BD3 and BD5 may have inherited a genetic susceptibility to the development of RP. Alternatively, their RP may have been triggered by the purported environmental factor which triggered SSc in the proband. In either case, whatever caused the development of RP in the sisters of the proband is implicated in the development of SSc in the patient herself.

An additional susceptibility factor may be required for the induction of SSc

Assuming that all the family members were indeed exposed to the same environmental trigger, the additional presence of a vascular defect in both BD3 and BD5 does not appear to have been sufficient to provoke the development of SSc. Therefore, the evidence suggests that an additional susceptibility factor was necessary, both for the induction of SSc, and for production of SSc-associated autoantibodies in the proband.

This would be consistent with the model of SSc proposed by Rosen *et al.* (288), in which RP was described as being a very important predisposing factor to the development of SSc (119,288). In Rosen's model, RP was proposed to interact with at least one other predisposing factor, namely abnormal metal ion status, to initiate the pathogenesis of SSc along with the production of particular SSc-associated autoantibodies. As reviewed in Section 1.7, this hypothesis was based on the previous study by this group in which it was demonstrated that some of the particular autoantigens recognized by dc-SSc sera could be specifically fragmented under conditions of oxidative stress by metal-ion catalysed processes (119,173). Metal ions have been shown to accumulate in the nucleolus, and severe RP is characterized by episodes of ischaemic reperfusion and the associated production of ROS. Significantly, the 70-kDa subunit of the U1 RNP particle was one of these antigens, along with topo I, the largest subunit of RNAP II, and NOR-90. (It should, however, be noted that, while the U1 RNP subunit was cleaved by Cu (II) ions, both topo I and the RNAP II subunit were most efficiently cleaved by Fe (II) ions (173), implying that the production of RNAP II/topo I fragments and the production of U1 RNP fragments are independent events, possibly occurring in separate groups of SSc patients. Those SSc sera with anti-RNAP II±topo I antibodies, and SSc sera with anti-U1 RNP antibodies do indeed seem

to represent different subgroups of patients (see Chapter 4)). Thus, given the production of anti-U1 RNP antibodies by patient BD1, there is reason to believe that Cu (II) ion-catalysed fragmentation reactions may have occurred as part of the aetiopathogenesis of SSc in patient BD1. This suggests that a metal ion abnormality occurring only in the proband may have been the additional factor required to instigate the complete disease process in this particular family member.

The probable role of an environmental factor in SSc has been suggested by a number of studies (97,109,137,173,293,295,683), including the present one, and Rosen *et al.* did, in fact, state that their model was likely to be incomplete (288). Noticeably, while their model included the occurrence of RP in SSc, together with a plausible mechanism leading to the production of SSc-associated autoantibodies, the possible involvement of an environmental factor was not addressed (although it could be that the proposed environmental factor *is* an abnormal exposure to metal ions (91,120)). Two other key features of SSc also require inclusion in any reasonable model of aetiopathogenesis, namely the proposed cycle of vascular damage (40,197,263), and the characteristic cutaneous fibrosis (199). In the case of the model described by Rosen *et al.*, it was suggested that the cycle of vascular damage arises via the generation of ROS. The metal abnormality was proposed to lower the threshold for vasospasm, substantially increasing ROS production. The ROS-induced damage was said to increase the frequency of ischaemic-reperfusion, with the consequence of a further increase in the generation of ROS. Hypoxia and ROS were proposed to promote the SSc-specific fibrotic phenotype, together with specific immune responses resulting from the ROS-induced autoantigen fragmentation events. These immune responses could, in turn, be responsible for inducing fibrogenic cytokine production (288).

However, other reasonable models of SSc have been suggested (40,105,164,199), and may also provide clues concerning the present results. For example, the vascular endothelial damage characteristic of SSc may be initiated by an environmental factor, rather than by ROS (293,195). Furthermore, as discussed in Section 1.3, there is strong evidence that, once endothelial damage has occurred, the cycle of damage in SSc is perpetuated by an immune-mediated mechanism (132,150,146,45). Also, the enrichment of certain HLA Class II and/or C4A null alleles in the SSc population should not be overlooked (25,33).

In the context of pathogenetic anti-EC autoantibodies, HLA alleles may also have been involved in the susceptibility of the proband to SSc

Thus, regarding the particular susceptibility of the proband to SSc, another possible predisposing factor necessary for disease induction in the proband could have been the presence of particular HLA alleles. As discussed in Chapter 3, given the same provoking factors, the mere presence of particular HLA alleles seems unlikely to explain the development of SSc in one member of a family but not in others, since

autoantibodies which recognize intracellular antigens are not thought to be involved in the pathogenesis of SSc. However, one particular type of autoantibody *has* recently been linked with a directly pathological response in SSc: namely anti-EC antibodies (45,29,266), which have been shown to be capable of inducing EC apoptosis (29). Assuming that anti-EC antibodies are involved in the pathogenesis of SSc, a feasible mechanism is suggested, by which alternative HLA alleles can actually affect disease induction. For the expression of anti-EC antibodies to occur, the production of unusual EC-derived fragments is likely to be necessary, in order to overcome tolerance. In this regard, there is evidence that granzyme-1 is involved in the production of EC-derived fragments in SSc, and that these are subsequently processed into cryptic epitopes (5). Raised levels of granzyme-1 are often found in SSc. Environmental agents may also be capable of inciting the production of neoantigens during endothelial damage. Other possibilities include the proposed metal ion abnormality, acting on certain EC antigens in association with ROS. In any case, some HLA alleles would be capable of efficiently presenting a given cryptic peptide, while others would not. Consequently, the production of anti-EC antibodies would occur only in the presence of appropriate HLA alleles. Subsequently, anti-EC antibodies may set up the cycle of immune-mediated vascular damage believed to be central to the pathogenesis of SSc (197,45). Meanwhile, individuals without appropriate genetic factors would, presumably, still have evidence of damage to the vascular endothelium. In support of this is the reported detection of Factor VIII-related antigen (a marker of EC damage) in 20% of the first-degree relatives of SSc patients (*c.f.* 62% of SSc patients) (159), which implies that vascular endothelial damage occurs in some family members of SSc patients, but that it is not propagated into an immune-mediated cycle of destruction with the consequent production of fibrogenic cytokines at the inflammatory perivascular site. Further evidence is provided by the study of Si-SSc by Rustin *et al.*, in which endothelial damage was observed not only in silica-exposed miners who developed Si-SSc, but also in a proportion of those who did not (293). This would also explain the observation that, in many multicase SSc families, the affected individuals tend to have similar HLA profiles (61,138,223,235,241,310). Indeed, in the previously reported case of conjugal SSc, the husband and wife both happened to share several HLA alleles, and the same inciting stimulus was implicated (56), which could indicate that the same cryptic endothelial antigens were produced in response to the same environmental stimulus.

To summarize, in the context of currently accepted models of the aetiopathogenesis of SSc, the present results strongly suggest that the same environmental insult has had an alternative denouement in the different family members, with only the proband responding in a pathological fashion, while some of her relatives appeared to possess a phenotypic and immunogenetic repertoire able to deal with and resolve the challenge with the minimum of clinical and autoimmune sequelae.

CHAPTER 6

DISCUSSION

6 DISCUSSION

6.1 General discussion

The central conundrum of the immunopathogenesis of SSc is implicit in the close association between disease expression and autoantibody specificity in individual patients. Thus, while some SSc-specific autoantibodies are demonstrably capable of inhibiting the cellular functions of the autoantigens which they recognize during *in vitro* studies, they are unlikely to have access to the intracellular locations of these antigens *in vivo*. Indeed there is no evidence that functional inhibition of the intracellular autoantigens recognized by SSc sera is a direct or indirect cause of the pathological changes characteristic of the disease.

A major area of progress was the identification of the key steps required for the production of activated autoreactive T and B cells. Such work has demonstrated a general requirement for the presentation of cryptic epitopes of an autoantigen by APCs, in order for tolerance to be broken at the level of T cells recognizing minor antigenic determinants. It has been shown that, once this has occurred, the subsequent activation of autoreactive B cells, which includes their function as APCs, can lead to the spreading of T cell responses to different epitopes of the antigen, and the eventual perpetuation of the immune response by the self antigen itself, as minor antigenic determinants become major determinants in the established immune response.

While a number of events may lead to an APC presenting sufficient quantities of a cryptic epitope to induce activation of naive autoreactive T cells, an inherent requirement is the provision of an altered form of the self-antigen (or of an antigen with which it is physically associated), or, alternatively, of a molecular mimic. Thus, it appears that the autoantigens recognized by SSc sera are seen by the immune system in an altered context, and, further, that the expression of SSc autoantibodies is disease-specific. It therefore follows that a more accurate description of the situation would be that the provision of certain self-antigens in an altered context is the truly disease-specific aspect of SSc immunopathogenesis. This alternative concept has been emerging in recent years, largely due to ground-breaking work of Rosen *et al.* (47-49,288) and, also, Utz *et al.* (347), and Casiano *et al.* (50). Working mainly in the area of SLE, such studies have provided a plethora of results, which demonstrate that separate groups of autoantigens commonly recognized by SLE sera are the subject of certain types of modification during apoptosis. Since deficiencies in apoptosis appear to be central to the development of at least some forms of SLE, it was suggested that these particular autoantibodies could be seen as the 'immunological footprints' of the aetiopathogenesis of SLE (47). More recently, the exciting observation by Casciola-Rosen *et al.* that metal-ion catalyzed oxidation reactions may result in the unusual fragmentation of SSc autoantigens in the nucleolus (46) has begun to extend the concepts described above for SLE, to include SSc. Thus, it was recently proposed by Rosen *et al.* (288) that the particular autoantibodies produced in autoimmune diseases

represent the '...immunological memory of the altered circumstances that initially revealed... ...cryptic structure...', and, furthermore, that these autoantibodies may be indicative of aberrant disease-specific pathological processes. The present study contributes to this developing view.

6.2 Main conclusions

The present study began by examining the frequency and specificity of ANAs and ANoAs in sera from SSc patients and from their family members. The correlation between the production of SSc-associated autoantibodies and the presence of SSc was striking, despite the fact that some relatives produced strong IF-ANoAs together with strong bands on IP gels. Therefore, the views of Rosen *et al.* (288), as described above, were strongly supported.

However, an increased frequency of IF-ANoAs in the first-degree relatives of SSc patients compared with normal controls indicated that a genetic factor may cause the nucleolus to become a focus of the autoimmune response in both SSc patients and their blood-relatives. This finding suggested that some, but not all, of the factors required for the induction of SSc may be present in the patients' family members. Based on the central tenet of Rosen's suggestion, as quoted above, it therefore follows that the autoantibodies recognized by the SSc-free relatives of SSc patients are, in effect, reporting on the scenario which lead to the presentation of cryptic epitopes of the autoantigens which they recognize. The importance of this conjecture is that by identifying these autoantigens significant aspects of the early pathogenesis of SSc may be elucidated. Furthermore, differences between the affected and unaffected members of a family may be uncovered as a result of such knowledge, leading, in turn, to identification of vital predisposing factors involved in the development of SSc in the proband. Important protective factors may also be revealed. As well as aiding the development of a full aetiopathogenic model of the disease, such insights may have consequences for effective treatment of this condition.

Next, the clinical associations of autoantibodies were studied in a larger cohort of SSc patients. Three major subgroups of SSc patients, each characterized by the presence of a particular, mutually exclusive, SSc-specific autoantibody (*viz.* anti-RNAP III, anti-topo I and ACA groups) were detected, thus confirming the results of Bunn *et al.* (36). Our results demonstrated that anti-RNAP II antibodies frequently accompany anti-topo I antibodies, as well as being produced by patients in the anti-RNAP III-defined group.

The recognition of RNAP subunits by different groups of anti-RNAP sera were then explored. When considered alongside the results of others, these studies suggested that anti-RNAP II antibodies can be produced in the context of two distinct immune response pathways, each with a characteristic pattern of epitope spread, and, possibly involving different initiating stimuli. This possibility would have important implications for the study of HLA associations with autoantibody subgroups, in that the

HLA alleles responsible for the production of a particular autoantibody would depend on the particular immune response pathway involved. This would not contradict the ideas of Rosen *et al.*, since each pathway leading to the production of a particular autoantibody would be representative of distinct pathological processes occurring in the relevant patient group.

It was further suggested that the subset of patients defined by the production of anti-RNAP II and/or topo I antibodies described here may correspond to the particular group of SSc patients alluded to by Casciola-Rosen *et al.*, who appear to have unique access to RNAP II and topo I antigens in an unusually fragmented form (46). If this particular connection was confirmed, it would suggest that distinct pathological processes are occurring in the three subgroups of SSc patients defined by the presence of a particular SSc-associated autoantibody, and would be in direct support of the separate disease model of Fanning *et al.* (86).

In further support of the model of Fanning *et al.* was the other main aspect of this part of the project. Several of the previously reported clinical associations of SSc-associated antibodies were confirmed, and it was convincingly demonstrated that the three main groups of autoantibodies found in SSc (i.e. anti-RNAP III, anti-topo I and ACAs) are each associated with varying degrees of cutaneous involvement, further supporting the distinction between these three groups of SSc patients. An important additional finding was the demonstration that anti-Ro and anti-La antibodies may be of significance to the pathogenesis of SSc, and thus may be prognostically useful in individual cases.

The final part of this project concerned the production of autoantibodies by several different members of a single family including the spouse of a U1 RNP-positive proband. This provided convincing circumstantial evidence that a combination of several genetic and environmental factors is involved in the aetiopathogenesis of SSc, as suggested by a number of authors (288,331). Bearing in mind the model of LeRoy (195) and the model of Rosen *et al.* (288) it appears possible that a total of six or more different factors may be required to be present for the development of SSc: vascular defects (as reflected by an inherited predisposition towards RP, with its episodes of ischaemic reperfusion and the generation of ROS), an environmental stimulus (which may initiate endothelial damage), the presence of abnormal metal ion status (which, as Rosen *et al.* propose, may lower the threshold for the induction of vascular spasms, and is responsible for the unique cleavage of SSc-associated autoantigens in association with ROS), complement deficiency (which may affect the efficient clearance of EC debris), the presence of particular HLA alleles (which allows propagation of environmentally induced endothelial damage into a cycle of immune-mediated endothelial damage, with the consequent production of a pro-inflammatory, pro-fibrotic perivascular environment), and, possibly, an unknown genetic factor which may make the nucleolus a focus for the immune response in SSc.

If it is accepted that the aetiopathogenesis of SSc does involve the converging influences of several different genetic and environmental factors, and, further, that distinct pathological processes are occurring in the three main serologically defined subgroups of SSc patients, it follows that particular combinations of some of these susceptibility factors may be responsible for the development of particular subgroups of disease. This stresses the importance of carefully defining patient subgroups when studying the aetiopathogenesis of SSc.

To summarize, the results presented here are consistent with the current model of aetiopathogenesis in which SSc-specific autoantibodies, while not being directly involved in disease pathogenesis, are, nonetheless, extremely reliable reporters of disease-specific pathological phenomena.

APPENDICES

Appendix I: Organ involvement severity scoring systems for SSc

Renal involvement

(0) No abnormalities: normal

(1) Mild: CC <60 mg/ml; SC 1.3-1.6 mg/dl; DBP 105-114 mmHg; UP 2+

(2) Moderate: SC 1.7-2.9 mg/dl; DBP >120 mmHg; UP 3-4+

(3) Severe: SC>3.0 mg/dl

(4) Endpoint: dialysis

Pulmonary involvement

(0) No abnormalities: normal

(1) Mild: DLCO 70-80%; FVC 70-80%; bibasilar dry rales/ interstitial fibrosis

(2) Moderate: DLCO 50-69%; FVC 50-69%

(3) Severe: DLCO<50%; FVC <50%

(4) Endpoint: Oxygen required

CC, creatinine clearance; SC, serum creatinine; DBP, diastolic blood pressure; UP, urinary protein; DLCO, carbon monoxide diffusion capacity; FVC, forced vital capacity

Appendix II: Centrifuge protocols

Protocol no.	Centrifuge model/rotor	Time (min)	Speed (r.p.m.)	Temperature (°C)
1	Hereaus, Labofuge 6000	10	3000	20
2	Beckman GPR/GH3.7	5	1500	1
3	Beckman GS-15R/F2402	20	12000	1
4	Beckman GPR/GH3.7	3	4000	1
5	MSE, Microcentaur	10 sec	13000	20
6	MSE, Microcentaur	2	13000	20
7	Hereaus, Biofuge Fresco	10	9000	1
8	Jouan, BR4i/S40	5-20*	4100	1
9	Jouan, BR4i/S40	4	3400	1

*as applicable, see text

Appendix III: Precautions taken for RNA work

1. Dimethyl pyrocarbonate-treated water was used to prepare all solutions (1 ml was added to each litre of Milli-Q water prior to autoclaving

2. All pipette tips and Eppendorf tubes were double-bagged and autoclaved prior to use

3. Clean, molecular biology-grade chemicals were always used, and were weighed by pouring directly into weighing boats lined with autoclaved aluminium foil

4. Tissue culture-grade plasticware was used for making up and storing solutions

5. Clean latex gloves were always worn

6. All electrophoresis equipment and staining trays were kept clean. Equipment was soaked in Decon-90 (or similar laboratory cleanser) and rinsed well in autoclaved Milli-Q water prior to use. Glass plates were also rinsed in 50% methanol: 50% nitric acid, and rinsed in autoclaved Milli-Q water prior to use.

7. The sonicator probe was well cleaned before use: the sonicator was switched on whilst the probe was fully immersed in 50 ml of each of the following solutions in turn:

(i) Autoclaved Milli-Q water
(ii) Decon-90
(iii) Autoclaved Milli-Q water
(iv) Autoclaved Milli-Q water
(v) 50% methanol: 50% nitric acid
(vi) Autoclaved Milli-Q water
(vii) Autoclaved Milli-Q water
(viii) Ethanol
(ix) Autoclaved Milli-Q water
(x) Autoclaved Milli-Q water
(xi) Autoclaved Milli-Q water

Appendix IV: List of families and family members studied

Sample	Diagnosis	Relationship	Sex
A1	lc-SSc	Proband	F
A2	-	Father	M
A3	-	Mother	F
A4	-	Brother	M
A5	-	Sister	F
A6	-	P.G.father	M
A8	-	M.G.mother	F
B 1	lc-SSc	Proband	F
B 4	-	Son	M
B 5	-	Daughter	F
B 6	-	Son	M
B 7	-	Spouse	M
B 8	-	Sister	F
B 9	-	Nephew	M
B10	-	Brother	M
B11	-	Brother	M
BA1	lc-SSc	Proband	F
BA2	-	Sister	F
BA3	-	T. sister	F
BB1	dc-SSc	Proband	F
BB2	-	Sister	F
BB3	-	Sister	F
BB4	-	Sister	F
BB5	-	Spouse	M
BB6	-	Mother	F
BB7	RA	Father	M
BC1	lc-SSc	Proband	F
BC2	-	Spouse	M
BC3	-	Daughter	F
BC4	SLE/Pm	Mother	F
BC5	RP	Father	M
BC6	RA	Sister	F
BD1	dc-SSc	Proband	F
BD2	-	Spouse	M
BD3	RP	Sister	F
BD5	RP	Sister	F
BD6	-	Son	M
BD7	-	Sister	F
BE 1	dc-SSc	Proband	F
BE 2	RP	Spouse	M
BE 3	RP	Daughter	F
BE 4	-	Son	M
BE 5	-	Brother	M
BE 6	RP	Mother	F
BE 7	RP	Sister	F
BE 8	RP	Sister	F
BE 9	-	Brother	M
BE10	RP	Niece	F
BE11	-	1st degree	NR
BE12	RP	Niece	F
BE13	RP	Niece	F
BE14	RP	Niece	F
BE15	-	Nephew	M
BE16	-	Nephew	M
BG1	lc-SSc	Proband	F
BG2	-	1st degree	NR
BG3	-	Spouse	M
BG4	-	Mother	F
E1	dc-SSc	Proband	F
E2	RP	Mother	F
E4	-	Brother	M
E6	-	Sister	F
E8	RP	Brother	M
E9	-	Sister	F
F1	lc-SSc	Proband	F
F2	RP	Mother	F
F3	-	Spouse	M
F4	-	Daughter	F
G1	lc-SSc	Proband	F
G2	-	Daughter	F
H1	dc-SSc	Proband	F
H2	-	Mother	F
H3	-	Father	M
H4	-	Daughter	F
H5	-	Son	M
I1	dc-SSc	Proband	M
I2	-	Spouse	F
I3	dc-SSc	Daughter	F
I4	-	G.daughter	F
I5	-	Spouse of I3	M
I6	-	Grandson	M
I7	-	Brother	M
K1	lc-SSc	Proband	F
K3	Juvenile arth.	Son	M
K4	-	Spouse	M
K5	-	Daughter	F
L1	lc-SSc	Proband	F
L3	-	Son	M
LA1	lc-SSc, d LE	Proband	M
LA2	-	Spouse	F
LA3	RP	Son	M
LA4	RP	Son	M
LA6	-	Father	M
LA7	RP	Brother	M
LA8	SLE	Brother	M
LA9	RP	Sister	F
LC1	dc-SSc	Proband	F
LC2	-	Mother	F
LC3	?Arthritis	Father	M
LC4	-	Sister	F
LD1	dc-SSc, mor.	Proband	F
LD2	-	Mother	F
LD4	-	Son	M
LE1	lc-SSc	Proband	F
LE2	RP	Mother	F
LE3	RP	Father	M
LE4	-	Sister	F
LE5	-	Brother	M
LG1	lc-SSc	Proband	F
LG2	RP	Son	M
LG3	-	Sister	F
LG4	-	Sister	F
LG5	RP	Sister	F
LJ 1	dc-SSc	Proband	F
LJ 2	-	Spouse	M
LJ 3	-	Son	M
LJ 4	-	Daughter	F
LJ 5	-	Son	M
LJ 6	-	Son	M
LJ 7	-	Mother	F
LJ 8	PA	Father	M
LJ 9	-	Sister	F
LJ10	RP	Brother	M
LK1	lc-SSc	Proband	F
LK2	-	Brother	M
LK3	-	Son	M
LK4	-	Spouse	M
LK5	-	Sister	F
LK6	-	Sister	F
LL1	lc-SSc, RA	Proband	F
LL3	-	Daughter	F
LL4	RA	Sister	F
LL7	-	Daughter	F
MA 1	lc-SSc	Proband	F
MA 2	-	Mother	F
MA 3	-	Brother	M
MA 4	RP	Sister	F
MA 6	-	Sister	F
MA 7	-	Brother	M
MA 8	-	Brother	M
MA10	RP in past	Sister	F
MA11	-	Daughter	F
MA12	-	Son	M
MA13	-	Spouse	M
MA15	PA	P.G.mother	F
MB1	dc-SSc	Proband	F
MB3	-	Son	M
MB4	-	Son	M
MC1	dc-SSc	Proband	F
MC2	-	Daughter	F
MC3	-	Son	M
MD 1	lc-SSc	Proband	F
MD 2	-	Sister	F
MD 3	-	Sister	F
MD 4	RP	Brother	M
MD 5	RP	Brother	M
MD 6	-	Mother	F
MD 7	RP	Father	M
MD 8	-	Spouse	M
MD 9	-	Son	M
MD10	-	Son	M
MI1	lc-SSc	Proband	F

Sample	Diagnosis	Relationship	Sex
MI2	-	Sister	F
MI3	-	Mother	F
MI4	-	Sister	F
MI5	-	Sister	F
MI6	-	Daughter	F
MI7	-	Spouse	M
MI9	RP	Son	M
MJ1	lc-SSc	Proband	F
MJ2	-	Son	M
MJ3	PA	Sister	F
MJ4	RP	Brother	M
MK1	dc-SSc	Proband	M
MK2	-	Daughter	F
MK3	RP	Daughter	F
MM1	lc-SSc	Proband	F
MN1	lc-SSc	Proband	F
MN2	-	Son	M
MN3	-	Son	M
MN4	Arthritis	Sister	F
MO1	lc-SSc	Proband	F
MO2	-	Son	M
MO4	RP	Brother	M
MO5	RP	Sister	F
MP1	lc-SSc	Proband	F
MP2	-	Father	M
MP4	RP in past	Mother	F
MP5	-	Brother	M
N 1	dc-SSc	Proband	F
N 2	-	Son	M
N 3	-	Id.T. daughter	F
N 4	-	Father	M
N 5	-	Sister	F
N 7	Hyperthyroid	Sister	F
N 8	-	Brother	M
N 9	-	Brother	M
N10	-	Id.T. Daughter	F
N11	-	Spouse	M
NA1	lc-SSc	Proband	F
NA2	-	1st degree	M
NA3	Mor., hyperthy.	1st degree	F
NAA1	lc-SSc	Proband	F
NAA2	lc-SSc	Sister	F
NAA3	-	Brother	M
NAA4	-	Father	M
NAA5	-	Son	M
NAA6	Ank. spond.	Son	M
NAA7	-	Daughter of NAA2	F
NAA9	-	Nephew	F
NAB1	dc-SSc	Proband	F
NAB2	-	1st degree	F
NAB3	-	1st degree	F
NAC1	dc-SSc	Proband	F
NAC2	-	1st degree	F
NAC3	SLE	1st degree	F
NAC4	-	1st degree	F
NR1	SSc	Proband	F
NR2	-	Sister	F
NR3	-	1st degree	NR
NR4	-	1st degree	NR
NRM1	lc-SSc	Proband	F
NRM2	lc-SSc	Daughter	F
NRM3	-	Grandson	M
NRM4	-	Grandson	M
NRM5	RP	Daughter	F
NRM6	-	Daughter	F
NRM7	RP	Son	M
NRT1	lc-SSc	Proband	F
NRT2	lc-SSc	Id.T.sister	F
NRT3	-	Daughter	F
NRT4	RP	Son of NRT2	M
O1	lc-SSc	Proband	F
O2	-	T. sister	F
O3	-	Sister	F
O5	-	Father	M
P1	lc-SSc	Proband	F
P2	-	Spouse	M
SA1	dc-SSc	Proband	F
SA2	-	Mother	F
SA4	-	Brother	M
SA5	RP	Brother	M
SB1	lc-SSc	Proband	F

Sample	Diagnosis	Relationship	Sex
SC1	lc-SSc	Proband	F
SC2	RP	Father	M
SC3	-	Sister	F
SC4	-	Mother	F
SC5	-	M.G.mother	F
SC6	-	M.G.father	M
SC7	RP	P.G.mother	F
SD1	lc-SSc	Proband	F
SD2	-	Son	M
SD3	-	Spouse	M
SD4	-	Sister	F
SD5	-	Sister	F
SE1	dc-SSc	Proband	F
SE2	RP	Son	M
SE3	-	Spouse	M
SE4	RP	Sister	F
SE5	RP	Sister	F
SE6	RP	Daughter	F
SF1	dc-SSc	Proband	F
SG1	dc-SSc	Proband	F
SG2	RP	Mother	F
SG6	-	Sister	F
SG7	-	Niece	F
SG8	RP	Niece	F
SH1	lc-SSc	Proband	F
SH2	-	Son	M
SH3	RP	Daughter	F
SH4	-	Spouse	M
SI1	lc-SSc	Proband	F
SI2	-	Spouse	M
SI3	-	Brother	M
SI4	-	Brother	M
SI5	-	Mother	F
SI6	-	Sister	F
T1	dc-SSc	Proband	F
T2	-	Mother	F
T3	-	Spouse	M
U1	dc-SSc	Proband	F
U2	-	Brother	M
U3	-	Brother	M
U4	-	Father	M
V 1	dc-SSc, PA	Proband	F
V 2	RP	Sister	F
V 3	RP	Son	M
V 4	-	Daughter	F
V 5	RP	Brother	M
V 6	-	Spouse	M
V 7	-	Son	M
V 8	Hyperthyroid	Sister	F
V 9	-	Brother	M
V10	-	Brother	M
W1	dc-SSc	Proband	F
W2	-	Brother	M
W3	-	Mother	F
W4	-	Father	M
X1	lc-SSc	Proband	F
X2	-	1st degree	F
X4	-	1st degree	M

Key:

lc-SSc, limited cutaneous SSc
dc-SSc, diffuse cutaneous SSc
RA, rheumatoid arthritis
SLE, systemic lupus erythematosus
Pm, polymyositis
RP, Raynaud's phenomenon
NR, not recorded
Arth., arthritis
dLE, discoid lupus erythematosus
Mor, morphea
PA, pernicious anaemia
hyperthy., hyperthyroid
Ank. spond., ankylosing spondylitis

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Abbas, A. K., A. H. Lichtman, and J. S. Pober. 1991. Cellular and molecular immunology. W.B. Saunders Company, Philadelphia.

2. Adams, D. 1996. How the immune system works and why it causes autoimmune diseases. *Immunol. Today* 17:300-302.

3. Alspaugh, M. A. and E. M. Tan. 1975. Antibodies to cellular antigens in Sjogren's syndrome. *J. Clin. Invest.* 55:1067-1073.

4. Amital, H. and Y. Schoenfeld. 1996. Nucleosomes, DNA and SLE: Where is the starting point? *Clin. Exp. Rheumatol.* 14:475-477.

5. Andrade, F., S. Roy, D. Nicholson, N. Thornberry, A. Rosen, and L. Casciola-Rosen. 1998. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. *Immunity* 8:451-460.

6. Arnett, F. C., R. F. Howard, F. Tan, J. M. Moulds, W. B. Bias, E. Durban, H. D. Cameron, G. Paxton, T. J. Hodge, P. E. Weathers, and J. D. Reveille. 1996. Increased prevalence of systemic sclerosis in a native American tribe in Oklahoma. Association with an Amerindian HLA haplotype. *Arthritis Rheum.* 39:1362-1370.

7. Arnett, F. C., J. D. Reveille, R. Goldstein, K. M. Pollard, K. Leaird, E. A. Smith, E. C. LeRoy, and M. J. Fritzler. 1996. Autoantibodies to fibrillarin in systemic sclerosis (scleroderma). An immunogenetic, serological, and clinical analysis. *Arthritis Rheum.* 39:1151-1160.

8. Artlett, C. M., C. M. Black, D. C. Briggs, C. Stephens, and K. I. Welsh. 1996. DNA allelic alterations within VNTR loci of scleroderma families. *Br. J. Rheumatol.* 35:1216-1222.

9. Artlett, C. M., C. M. Black, D. C. Briggs, C. O. Stevens, and K. I. Welsh. 1996. Telomere reduction in scleroderma patients: a possible cause for chromosome instability. *Br. J. Rheumatol.* 35:732-737.

10. Atkinson, J. P. 1989. Complement deficiency: predisposing factor to autoimmune syndromes. *Clin. Exp. Rheumatol.* 7 (Suppl.3):95-101.

11. Atkinson, J. P. 1995. Some thoughts on autoimmunity. *Arthritis Rheum.* 38:301-305.

12. Barlow, A. K., X. He, and C. Janeway,Jr. 1998. Exogenously provided peptides of a self-antigen can be processed into forms that are recognized by self-T cells. *J. Exp. Med* 187:1403-1415.

13. Barnett, A. J. and L. J. McNeilage. 1993. Antinuclear antibodies in patients with scleroderma (systemic sclerosis) and in their blood relatives and spouses. *Ann. Rheum. Dis.* 52:365-368.

14. Beck, J. S., J. R. Anderson, K. G. Gray, and N. R. Rowell. 1963. Antinuclear and precipitating autoantibodies in progressive systemic sclerosis. *The Lancet* December 7:1188-1190.

15. Behar, S. M. and S. A. Porcelli. 1995. Mechanisms of autoimmune disease induction. The role of the immune response to microbial pathogens. *Arthritis Rheum.* 38:458-476.

16. Belch, J. J. F. and C. Maple. 1995. Raynaud's phenomenon. In Connective Tissue Diseases. J. J. F. Belch and R. B. Zurier, editors. Chapman & Hall, London. 131-150.

17. Bell, S., T. Krieg, and M. Meurer. 1989. Antibodies to Ro/SSA detected by ELISA: Correlation with clinical features in systemic scleroderma. *Br. J. Dermatol.* 121:35-41.

18. Bell, S. A., H. Faust, J. Mittermuller, H. J. Kolb, and M. Meurer. 1996. Specificity of antinuclear antibodies in scleroderma-like graft-versus-host disease: clinical correlation and histocompatibility locus antigen association. *Br. J. Dermatol.* 134:848-854.

19. Bernardi, R., C. Negri, M. Donzelli, F. Guano, M. Torti, E. Proseri, and A. I. Scovassi. 1995. Activation of poly(ADP-ribose) polymerase in apoptotic human cells. *Biochimie* 77:378-384.

20. Bernstein, R. M., J. C. Steigerwald, and E. M. Tan. 1982. Association of antinuclear and antinucleolar antibodies in progressive systemic sclerosis. *Clin. exp. Immunol.* 48:43-51.

21. Bianchi, D. W., G. K. Zickwolf, G. J. Weil, S. Sylvester, and M. A. DeMaria. 1996. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc. Natl. Acad. Sci. USA* 93:705-708.

22. Black, C. and D. A. Isenberg. 1992. Mixed connective tissue disease - goodbye to all that. *Br. J. Rheumatol.* 31:695-700.

23. Black, C. M. 1990. Systemic sclerosis: is there a treatment yet? *Ann. Rheum. Dis.* 49:735-737.

24. Black, C. M. and C. Stephens. 1993. Systemic sclerosis (scleroderma) and related disorders. In Oxford Textbook of Rheumatology. Oxford University Press, Oxford. 771-788.

25. Black, C. M., K. I. Welsh, P. J. Maddison, M. I. V. Jayson, and R. Bernstein. 1984. HLA antigens, autoantibodies and clinical subsets in scleroderma. *Br. J. Rheumatol.* 23:267-271.

26. Blaszczyk, M., M. Jarzabek-Chorzelska, S. Jablonska, T. Chorzelski, Z. Kolacinska-Strasz, E. H. Beutner, and V. Kumar. 1990. Autoantibodies to nucleolar antigens in systemic scleroderma: clinical correlations. *Br. J. Dermatol.* 123:421-430.

27. Bocchieri, M. H. and S. A. Jimenez. 1990. Animal models of fibrosis. *Rheum. Dis. Clin. N. Amer.* 16:153-167.

28. Bockenstedt, L. K., R. J. Gee, and M. J. Mamula. 1995. Self-peptides in the initiation of lupus autoimmunity. *J. Immunol.* 154:3516-3524.

29. Bordron, A., M. Dueymas, Y. Levy, C. Jamin, J. -P. Leroy, J. -C. Piette, Y. Shoenfeld, and P. Y. Youinou. 1998. The binding of some human antiendothelial cell antibodies induces endothelial cell apoptosis. *J. Clin. Invest.* 101:2029-2035.

30. Bouffard, P., M. -A. Laniel, and G. Boire. 1996. Anti-Ro(SSA) antibodies: clinical significance and biological relevance. *J. Rheumatol.* 23:1838-1841.

31. Brentnall, T. J., D. Kenneally, A. J. Barnatt, H. J. de Aizpurua, S. J. Lolait, R. Ashcroft, and B. H. Toh. 1982. Autoantibodies to fibroblasts in scleroderma. *J. Clin. Lab. Immunol.* 8:9-12.

32. Briggs, D. C., R. W. Vaughan, K. I. Welsh, A. Myers, R. M. duBois, and C. M. Black. 1991. Immunogenetic prediction of pulmonary fibrosis in systemic sclerosis. *The Lancet* 338:661-662.

33. Briggs, D. C., K. Welsh, R. S. Pereira, and C. M. Black. 1986. A strong association between null alleles at the C4A locus in the major histocompatibility complex and systemic sclerosis. *Arthritis Rheum.* 29:1274-1277.

34. Buhler, J. -M., J. Huet, K. E. Davies, A. Sentenac, and P. Fromageot. 1980. Immunological studies of yeast nuclear RNA polymerases at the subunit level. *J. Biol. Chem.* 255:9949-9954.

35. Bunn, C. C., D. Abrahams, C. Knight, and C. M. Black. 1998. Anti-fibrillarin (U3-RNP) in systemic sclerosis identifies young patients with myositis and pulmonary hypertension. *Br. J. Rheumatol.* 37:S60.(Abstr.)

36. Bunn, C. C., C. P. Denton, X. Shi-Wen, C. Knight, and C. M. Black. 1998. Anti-RNA polymerases and other autoantibody specificities in systemic sclerosis. *Br. J. Rheumatol.* 37:15-20.

37. Burns, C. J., T. J. Laing, B. W. Gillespie, S. G. Heeringa, K. H. Alcser, M. D. Mayes, M. C. M. Wasko, B. C. Cooper, D. H. Garabrant, and D. Schottenfeld. 1996. The epidemiology of scleroderma among women: assassment of risk from exposure to silicone and silica. *J. Rheumatol.* 23:1904-1911.

38. Cadena, D. L. and M. E. Dahmus. 1987. Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. *J. Biol. Chem.* 262:12468-12474.

39. Cairns, J. A. and A. F. Walls. 1997. Mast cell tryptase stimulates the synthesis of type I collagen in human lung fibroblasts. *J. Clin. Invest.* 99:1313-1321.

40. Campbell, P. M. and E. C. LeRoy. 1975. Pathogenesis of systemic sclerosis: a vascular hypothesis. *Sem. Arth. Rheum.* 4:351-368.

41. Carbone, F. R., C. Kurts, S. R. M. Bennett, J. F. A. P. Miller, and W. R. Heath. 1998. Cross-presentation: a general mechanism for CTL immunity and tolerance. *Immunol. Today* 19:368-373.

42. Carpentier, P. H. and H. R. Maricq. 1990. Microvasculature in systemic sclerosis. *Rheum. Dis. Clin. N. Amer.* 16:75-91.

43. Carroll, M. C. 1998. The lupus paradox. *Nature Gen.* 19:3-4.

44. Carson, D. A. 1994. The value of epitope mapping in autoimmune diseases. *J. Clin. Invest.* 94:1713.

45. Carvalho, D., C. O. S. Savage, C. M. Black, and J. D. Pearson. 1996. IgG antiendothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells in vitro. *J. Clin. Invest.* 97:111-119.

46. Casciola-Rosen, L., F. Wigley, and A. Rosen. 1997. Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: implications for pathogenesis. *J. Exp. Med.* 185:71-79.

47. Casciola-Rosen, L. A., G. Anhalt, and A. Rosen. 1994. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J. Exp. Med.* 179:1317-1330.

48. Casciola-Rosen, L. A., G. J. Anhalt, and A. Rosen. 1995. DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J. Exp. Med.* 182:1625-1634.

49. Casciola-Rosen, L. A., D. K. Miller, G. J. Anhalt, and A. Rosen. 1994. Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. *J. Biol. Chem.* 269:30757-30760.

50. Casiano, C. A., S. J. Martin, D. R. Green, and E. M. Tan. 1996. Selective cleavage of nuclear autoantigens during CD95 (Fas/APO-1)-mediated T cell apoptosis. *J. Exp. Med.* 184:765-770.

51. Casiano, C. A. and E. T. Tan. 1996. Recent developments in the understanding of antinuclear antibodies. *Int. Arch. Allergy Immunol.* 111:308-313.

52. Catoggio, L. J., R. M. Bernstein, C. M. Black, G. R. V. Hughes, and P. J. Maddison. 1983. Serological markers in progressive systemic sclerosis: clinical correlations. *Ann. Rheum. Dis.* 42:23-27.

53. Catoggio, L. J., R. P. Skinner, and P. J. Maddison. 1983. Frequency and clinical significance of anticentromere and anti Scl-70 antibodies in an English connective tissue disease population. *Rheumatol. Int.* 3:19-21.

54. Charlton, B. and K. J. Lafferty. 1995. The Th1/Th2 balance in autoimmunity. *Curr. Opin. Immunol.* 7:793-798.

55. Christner, P. J., J. Peters, D. Hawkins, L. D. Siracusa, and S. A. Jimenez. 1995. The tight skin 2 mouse. An animal model of scleroderma displaying cutaneous fibrosis and mononuclear cell infiltration. *Arthritis Rheum.* 38:1791-1798.

56. Christy, W. C. and G. P. Rodnan. 1984. Conjugal progressive systemic sclerosis (scleroderma): report of the disease in husband and wife. *Arthritis Rheum.* 27:1180-1182.

57. Cleland, L. G., D. A. Bell, M. Willans, and B. C. Saurino. 1978. Familial lupus. Family studies of HLA and serological findings. *Arthritis Rheum.* 21:183-191.

58. Clements, P. J., P. A. Lachenbruch, S. C. NG, M. Simmons, M. Sterz, and D. E. Furst. 1990. Skin score. A semiquantitative measure of cutaneous involvement that improves prediction of prognosis in systemic sclerosis. *Arthritis Rheum.* 33:1256-1263.

59. Cochran, W. G. 1954.*Biometrics* 10:417.

60. Conrad, K., G. Stahnke, B. Liedvogel, J. Mehlhorn, J. Barth, C. Blasum, P. Altmeyer, N. Sonnichsen, and K. -H. Frank. 1995. Anti-CENP-B response in sera of uranium miners exposed to quartz dust and patients with possible development of systemic sclerosis (scleroderma). *J. Rheumatol.* 22:1286-1294.

61. Cook, N. J., A. J. Silman, J. Propert, and M. I. D. Cawley. 1993. Features of systemic sclerosis (scleroderma) in an identical twin pair. *Br. J. Rheumatol.* 32:926-928.

62. Cook, R. G. 1994. Amino acid sequence analysis of blotted proteins. In Protein blotting. A practical approach. B. S. Dunbar, editor. IRL Press at Oxford University Press, New York. 207-20.

63. Cooper, G. S., M. A. Dooley, E. L. Treadwell, E. W. St.Clair, C. G. Parks, and G. S. Gilkeson. 1998. Hormonal, environmental, and infectious risk factors for developing systemic lupus erythematosis. *Arthritis Rheum.* 41:1714-1724.

64. Corcos, J. M., W. C. Robbins, B. Rogoff, and R. Heimer. 1961. Some serum protein abnormalities in patients with progressive systemic sclerosis and their relatives. *Arthritis Rheum.* 4:107.

65. Corden, J. L., D. L. Cadena, J. M. Ahearn, and M. E. Dahmus. 1985. A unique structure at the carboxyl terminal of the largest subunit of the eukaryotic RNA polymerase II. *Proc. Natl. Acad. Sci. USA* 82:7934-7938.

66. Craft, J., T. Mimori, T. L. Olsen, and J. A. Hardin. 1988. The U2 small nuclear ribonucleoprotein particle as an autoantigen. Analysis with sera from patients with overlap syndromes. *J. Clin. Invest.* 81:1716-1724.

67. Crow, M. K. and S. M. Friedman. Cellular immunology. In Rheumatology. 5.1-5.10.

68. Cuellar, M. L., E. Scopelitis, S. A. Tenenbaum, R. F. Garry, L. H. Silveira, G. Cabrera, and L. R. Espinoza. 1995. Serum antinuclear antuibodies in women with silicone breast implants. *J. Rheumatol.* 22:236-240.

69. Cutolo, M., A. Sulli, B. Seriolo, S. Accardo, and A. T. Masi. 1995. Estrogens, the immune response and autoimmunity. *Clin. Exp. Rheumatol.* 13:217-226.

70. de Crombrugghe, B., T. Vuorio, and G. Karsenty. 1990. Control of type I collagen genes in scleroderma and normal fibroblasts. *Rheum. Dis. Clin. N. Amer.* 16:109-123.

71. de Juan, M. D., J. Belzunegui, I. Belmonte, J. Barado, M. Figueroa, J. Cancio, S. Vidal, and E. Cuadrado. 1994. An immunogenetic study of familial scleroderma. *Ann. Rheum. Dis.* 53:614-617.

72. de Murcia, G., D. L. Cadena, J. M. Ahearn, and M. E. Dahmus. 1985. A unique structure at the carboxyl terminal of the largest subunit of the eukaryotic RNA polymerase II. *Proc. Natl. Acad. Sci. USA* 82:7934-7938.

73. Deguchi, Y. 1992. Spontaneous increase of transforming growth factor beta production by bronchoalveolar mononuclear cells of patients with systemic autoimmune diseases affecting the lung. *Ann. Rheum. Dis.* 51:362-365.

74. DeHoratius, R. J., R. Pillarisetty, R. P. Messner, and N. Talal. 1975. Anti-nucleic acid antibodies in systemic lupus erythematosus patients and their families. *J. Clin. Invest.* 56:1149-1154.

75. Denton, C. P., X. Shiwen, K. I. Welsh, J. D Pearson, and C. M. Black. 1996. Scleroderma fibroblast phenotype is modulated by endothelial cell co-culture. *J. Rheumatol.* 23:633-638.

76. Dick, T., R. Mierau, R. Sternfeld, E. M. Weiner, and E. Genth. 1995. Clinical relevance and HLA association of autoantibodies against the nucleolus organizer region (NOR-90). *J. Rheumatol.* 22:67-72.

77. Douvas, A. 1988. Does Scl-70 modulate collagen production in systemic sclerosis? *The Lancet* August 27:475-477.

78. Drapkin, R., M. Achten, and E. M. Tan. 1979. Identification of a nuclear protein (Scl-70) as a unique target of human antinuclear antibodies in scleroderma. *J. Biol. Chem.* 254:10514.

79. Dustoor, M. M., M. M. McInerney, D. J. Mazanec, and M. K. Cathcart. 1987. Abnormal lymphocyte function in scleroderma: A study on identical twins. *Clin. Immunol. Immunopathol.* 44:20-30.

80. Dvir, A., S. R. Peterson, M. W. Knuth, H. Lu, and W. S. Dynan. 1992. Ku autoantigen is the regulatory component of a template-associated protein kinase that phosphorylates RNA polymerase II. *Proc. Natl. Acad. Sci. USA* 89:11920-11924.

81. Earnshaw, W. C., P. S. Machlin, B. J. Bordwell, N. F. Rothfield, and D. W. Cleveland. 1987. Analysis of anticentromere autoantibodies using cloned autoantigen CENP-B. *Immunology* 84:4979-4983.

82. Earnshaw, W. C. and N. Rothfield. 1985. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma* 91:313-321.

83. Elkon, K. 1994. Apoptosis and autoimmunity. *ACR Basic Science Symp.* 58th AGM:(Abstr.)

84. Fagundus, D. M. and E. C. LeRoy. 1995. Systemic Sclerosis (Scleroderma). In Connective Tissue Diseases. J. J. F. Belch and R. B. Zurier, editors. Chapman & Hall Medical, London. 51-64.

85. Falkner, D., J. Wilson, T. A. Medsger,Jr, and P. A. Morel. 1998. HLA and clinical associations in systemic sclerosis patients with anti-Th/To antibodies. *Arthritis Rheum.* 41:74-80.

86. Fanning, G. C., K. I. Welsh, C. Bunn, R. Du Bois, and C. M. Black. 1998. HLA associations in three mutually exclusive autoantibody subgroups in UK systemic sclerosis patients. *Br. J. Rheumatol.* 37:201-207.

87. Fatenejad, S., W. Brooks, A. Schwartz, and J. Craft. 1994. Pattern of anti-small nuclear ribonucleoprotein antibodies in MRL/Mp-*lpr/lpr* mice suggests that the intact U1 snRNP particle is their autoimmunogenic target. *J. Immunol.* 152:5523-5531.

88. Fatenejad, S. and J. Craft. 1996. Intrastructural help in the diversification of humoral autoimmune responses. *Clin. exp. Immunol.* 106:1-4.

89. Fatenejeid, S., M. J. Mamula, and J. Craft. 1993. Intermolecular intrastructural B- and T-cell determinants in the diversification of autoantibodies to ribonucleoprotein particles. *Proc. Natl. Acad. Sci. USA* 90:12010-12014.

90. Fennell, R. H.,Jr, M. J. Maclachlan, and G. P. Rodnan. 1962. The occurrence of antinuclear factors in the sera of relatives of patients with systemic rheumatic disease. *Arthritis Rheum.* 5:296.

91. Ferri, C., L. Bernini, R. Cecchetti, A. Latorraca, G. Marotta, G. Pasero, R. Neri, and S. Bombardieri. 1991. Cutaneous and serological subsets of systemic sclerosis. *J. Rheumatol.* 18:1826-1832.

92. Fisher, R. A. and F. Yates. 1974. Statistical tables for biological, agricultural and medical research. Longmans, London.

93. Fleischmajer, R., J. S. Perlish, and J. R. T. Reeves. 1977. Cellular infiltrates in scleroderma skin. *Arthritis Rheum.* 20:975-984.

94. Fleischmann, G., G. Pflugfelder, E. K. Steiner, K. Javaherian, G. C. Howard, and J. C. Wang. 1984. *Drosophila* DNA topoisomerase I is associated with transcriptionally active regions of the genome. *Proc. Natl. Acad. Sci. USA* 81:6958-6962.

95. Flores, R. H., M. B. Stevens, and F. C. Arnett. 1984. Familial occurrence of progressive systemic sclerosis and systemic lupus erythematosus. *J. Rheumatol.* 11:321-323.

96. Forman, M. S., M. Nakamura, T. Mimori, C. Gelpi, and J. A. Hardin. 1985. Detection of antibodies to small nuclear ribonucleoproteins and small cytoplasmic ribonucleoproteins using unlabeled cell extracts. *Arthritis Rheum.* 28:1356-1361.

97. Frank, K. -H., M. Fussel, K. Conrad, H. -P. Rihs, R. Koch, B. Gebhardt, and J. Mehlhorn. 1998. Different distribution of HLA Class II and tumor necrosis factor alleles (TNF-308.2, TNFa2 microsatellite) in anti-topoisomerase I responders among scleroderma patients with and without exposure to quarz/metal dust. *Arthritis Rheum.* 41:1306-1311.

98. Frank, R., T. Giese, R. Dummer, T. Walther, M. Rytter, V. Ziegler, and U. F. Haustein. 1993. Silica-induced cytokine release in human monocyte cultures and its possible involvement in the pathophysiology of silica-associated scleroderma. *Eur. J. Dermatol.* 3:304-309.

99. Frayha, R. A., K. F. Tabbara, and R. S. Geha. 1977. Familial CRST syndrome with sicca complex. *J. Rheumatol.* 4:53-58.

100. Freedman, R. R. and M. D. Mayes. 1996. Familial aggregation of primary Raynaud's disease. *Arthritis Rheum.* 39:1189-1191.

101. Fritzler, M. J., C. A. von Muhlen, S. M. Toffoli, H. L. Staub, and R. M. Laxer. 1995. Autoantibodies to the nucleolar organizer antigen NOR-90 in children with systemic rheumatic diseases. *J. Rheumatol.* 22:521-524.

102. Fronek, Z., L. A. Timmerman, C. A. Alper, B. H. Hahn, K. Kalunian, B. M. Peterlin, and H. O. McDevitt. 1990. Major histocompatibility complex genes and susceptability to systemic lupus erythematosis. *Arthritis Rheum.* 33:1542-1553.

103. Fujii, T., T. Mimori, and M. Akizuki. 1996. Detection of autoantibodies to nucleolar transcription factor NOR 90/h UBF in sera of patients with rheumatic diseases, by recombinant autoantigen-based assays. *Arthritis Rheum.* 39:1313-1318.

104. Fujimoto, M., M. Shimozuma, N. Yazawa, M. Kubo, H. Ihn, S. Sato, T. Tamaki, K. Kikuchi, and K. Tamaki. 1997. Prevalence and clinical relevance of 52-kDa and 60-kDa Ro/SS-A autoantibodies in Japanese patients with systemic sclerosis. *Ann. Rheum. Dis.* 56:667-670.

105. Furst, D. E. and P. J. Clements. 1997. Hypothesis for the pathogenesis of systemic sclerosis. *J. Rheumatol.* 24 (suppl.48):53-57.

106. Fuss, I. J., W. Strober, J. K. Dale, S. Fritz, G. R. Pearlstein, J. M. Puck, M. J. Lenardo, and S. E. Straus. 1997. Characteristic T helper 2 T cell cytokine abnormalities in autoimmune lymphoproliferative syndrome, a syndrome marked by defective apoptosis and humoral autoimmunity. *J. Immunol.* 158:1912-1918.

107. Gammon, G. and E. Sercatz. 1989. How some T cells escape tolerance. *Nature* 342:183-185.

108. Gapin, L., Y. B. de Alba, A. Casrouge, J. P. Cabaniols, P. Kourilsky, and J. Kanellopoulis. 1998. Antigen presentation by dendritic cells focuses T cell responses against immunodominant peptides: Studies in the hen egg-white lysozyme (HEL) model. *J. Immunol.* 160:1555-1564.

109. Garcia-Zamalloa, A. M., E. Ojeda, C. Gonzalez-Beneitez, J. Goni, and A. Garrido. 1994. Systemic sclerosis and organic solvents: early diagnosis in industry. *Ann. Rheum. Dis.* 53:618-620.

110. Gelpi, C., E. Kanterewicz, J. Gratacos, I. N. Targoff, and J. L. Rodriguez-Sanchez. 1996. Coexistence of two antisynthetases in a patient with the antisynthetase syndrome. *Arthritis Rheum.* 39:692-697.

111. Gendi, N. S., K. I. Welsh, W. J. van Venrooij, R. Vancheeswaran, J. Gilroy, and C. M. Black. 1995. HLA type as a predictor of mixed connective tissue disease differentiation. Ten-year clinical and immunogenetic followup of 46 patients. *Arthritis Rheum.* 38:259-266.

112. Genth, E., R. Mierau, P. Genetzky, C. A. von Muhlen, S. Kaufmann, H. von Wilmowsky, M. Meurer, T. Krieg, H. -J. Pollmann, and P. W. Hartl. 1990. Immunogenetic associations of scleroderma-related antinuclear antibodies. *Arthritis Rheum.* 33:657-665.

113. Giacomelli, R., M. Matucci-Cerinic, P. Cipriani, I. Ghersetich, R. Lattanzio, A. Pavan, A. Pignone, M. L. Cagnoni, T. Lotti, and G. Tonietti. 1998. Circulating Vδ1+ T cells are activated and accumulate in the skin of systemic sclerosis patients. *Arthritis Rheum.* 41:327-334.

114. Giordano, M., G. Valentini, S. Migliaresis, U. Picillo, and M. Vatti. 1986. Different antibody patterns and different prognoses in patients with scleroderma with various extent of skin sclerosis. *J. Rheumatol.* 13:911-916.

115. Gladman, D. D., E. C. Keystone, M. Baron, P. Lee, D. Cane, and H. Mervert. 1981. Increased frequency of HLA-DR5 in scleroderma. *Arthritis Rheum.* 24:854-856.

116. Golan, T. D., K. B. Elkon, A. E. Gharavi, and J. G. Krueger. 1992. Enhanced membrane binding of autoantibodies to cultured keratinocytes of systemic lupus erythematosus patients after ultraviolet B/ultraviolet A irradiation. *J. Clin. Invest.* 90:1067-1076.

117. Gray, R. G. and R. D. Altman. 1977. Progressive systemic sclerosis in a family. Case report of a mother and son and review of the literature. *Arthritis Rheum.* 20:35-41.

118. Gregersen, P. K. 1993. Discordance for autoimmunity in monozygotic twins. Are "identical" twins really identical? *Arthritis Rheum.* 36:1185-1192.

119. Greidinger, E. L., L. Casciola-Rosen, and A. Rosen. 1998. Immune recognition of an oxidatively modified form of the U1-70kDa antigen is associated with Raynaud's phenomenon. *Arthritis Rheum.* 41:S323.(Abstr.)

120. Griem, P. and E. Gleichmann. 1995. Metal ion induced autoimmunity. *Curr. Opin. Immunol.* 7:831-838.

121. Griem, P., M. Wulferink, B. Sachs, J. B. Gonzalez, and E. Gleichmann. 1998. Allergic and autoimmune reactions to xenobiotics: how do they arise? *Immunol. Today* 19:133-141.

122. Gruschwitz, M. S., O. P. Hornstein, and P. von den Driesch. 1995. Correlation of soluble adhesion molecules in the peripheral blood of scleroderma patients with their in situ expression and with disease activity. *Arthritis Rheum.* 38:184-189.

123. Guery, J. -C. and L. Adorini. 1995. Dendritic cells are the most efficient in presenting endogenous naturally processed self-epitopes to Class II-restricted T cells. *J. Immunol.* 154:536-544.

124. Guldner, H. -H., C. Szostecki, H. -P. Vosberg, H. -J. Lakomek, E. Penner, and F. A. Bautz. 1986. Scl 70 autoantibodies from scleroderma patients recognize a 95 kDa protein identified as DNA topoisomerase I. *Chromosoma* 94:132-138.

125. Hardin, J. A. 1986. The lupus autoantigens and the pathogenesis of systemic lupus erythematosus. *Arthritis Rheum.* 29:457-460.

126. Harvey, G. and N. J. McHugh. 1994. Anti-RNA polymerase antibodies in systemic sclerosis: co-precipitation of RNA polymerase-II and topoisomerase-1. *Arthritis Rheum.* 37 (Suppl.):(Abstr.)

127. Harvey, G. R., C. Black, P. Maddison, and N. McHugh. 1996. Characterization of antinucleolar antibody reactivity in patients with systemic sclerosis and their relatives. *J. Rheumatol.* 24:477-484.

128. Harvey, G. R., A. L. Rands, and N. J. McHugh. 1997. Anti-RNA polymerase antibodies in systemic sclerosis (SSc): association with anti-topoisomerase I antibodies and identification of autoreactive subunits of RNA polymerase II. *Clin. exp. Immunol.* 105:468-474.

129. Harvey, G. R., A. L. Rands, Y. P. Patel, P. J. Maddison, and N. J. McHugh. 1996. Antibodies to RNA polymerases in systemic sclerosis: clinical and serological associations. *Arthritis Rheum.* 39 (Suppl.):S231.(Abstr.)

130. Hasegawa, M., M. Fujimoto, K. Kikuchi, and K. Takehara. 1997. Elevated serum tumor necrosis factor-alpha levels in patients with systemic sclerosis: association with pulmonary fibrosis. *J. Rheumatol.* 24:663-665.

131. Haug, B. L., J. S. Lee, and J. T. Sibley. 1994. Altered poly(ADP-ribose) metabolism in family members of patients with systemic lupus erthematosus. *J. Rheumatol.* 21:851-856.

132. Hawrylko, E., A. Spertus, C. A. Mele, N. Oster, and M. Frieri. 1991. Increased interleukin-2 production in response to human type I collagen stimulation in patients with systemic sclerosis. *Arthritis Rheum.* 34:580-587.

133. Haynes, D. C. and M. E. Gerschwin. 1982. The immunopathology of progressive systemic sclerosis (PSS). *Sem. Arth. Rheum.* 11:331-351.

134. Heath, W. R., C. Kurts, J. F. A. P. Miller, and F. R. Carbone. 1998. Cross-tolerance: A pathway for inducing tolerance to peripheral tissue antigens. *J. Exp. Med.* 187:1549-1553.

135. Herrick, A. L., K. Illingworth, A. Blann, C. R. M. Hay, S. Hollis, and M. I. V. Jayson. 1996. Von Willebrand factor, thrombomodulin, thromboxane, β-thromboglobulin and markers of fibrinolysis in primary Raynaud's phenomenon and systemic sclerosis. *Ann. Rheum. Dis.* 55:122-127.

136. Hershberg, R. M., D. H. Cho, A. Youakim, M. B. Bradley, J. S. Lee, P. E. Framson, and G. T. Nepom. 1998. Highly polarized HLA class II antigen processing and presentation by human intestinal epithelial cells. *J. Clin. Invest.* 102:792-803.

137. Hertzman, P. A. and I. A. Borda. 1993. The toxic oil syndrome and the eosinophilia-myalgia syndrome: pursuing clinical parallels. *J. Rheumatol.* 20:1707-1710.

138. Hietarinta, M., S. Koskimies, O. Lassila, E. Soppi, and A. Toivanen. 1993. Familial scleroderma: HLA antigens and autoantibodies. *Br. J. Rheumatol.* 32:336-338.

139. Hietarinta, M., R. Tertti, and O. Lassila. 1993. A patient with systemic sclerosis, severe cytopenias and the simultaneous presence of anti-centromere and anti-SCL-70-antibodies. *Clin. Exp. Rheumatol.* 11:457-461.

140. Higley, H., K. Persichitte, S. Chu, W. Waegell, R. Vancheeswaran, and C. Black. 1994. Immunocytochemical localization and serological detection of transforming growth factor β1. Association with type I procollagen and inflammatory cell markers in diffuse and limited systemic sclerosis, morphea, and Raynaud's phenomenon. *Arthritis Rheum.* 37:278-288.

141. Hill, M. B., J. L. Phipps, R. J. Cartwright, A. M. Ward, M. Greaves, and P. Hughes. 1996. Antibodies to membranes of endothelial cells and fibroblasts in scleroderma. *Clin. exp. Immunol.* 106:491-497.

142. Hirakata, M., J. Kanungo, A. Suwa, Y. Takeda, J. Craft, and J. A. Hardin. 1996. Autoimmunity to RNA polymerase II is focused at the carboxyl terminal domain of the large subunit. *Arthritis Rheum.* 39:1886-1891.

143. Hirakata, M., T. Mimori, M. Akizuki, J. Craft, J. A. Hardin, and M. Homma. 1992. Autoantibodies to small nuclear and cytoplasmic ribonucleoproteins in Japanese patients with inflammatory muscle disease. *Arthritis Rheum.* 35:449-456.

144. Hirakata, M., Y. Okano, U. Pati, A. Suwa, T. A. Medsger,Jr, J. A. Hardin, and J. Craft. 1993. Identification of autoantibodies to RNA polymerase II. Occurrence in systemic sclerosis and association with autoantibodies to RNA polymerases I and III. *J. Clin. Invest.* 91:2665-2672.

145. Hochberg, M. C., D. L. Perlmutter, T. A. Medsger,Jr, K. Nguyen, V. Steen, M. H. Weisman, B. White, and F. M. Wigley. 1996. Lack of association between augmentation mammoplasty and systemic sclerosis (scleroderma). *Arthritis Rheum.* 69:1125-1131.

146. Holt, C. M., N. Lindsey, J. Moult, R. G. Malia, M. Greaves, A. Hume, N. R. Rowell, and P. Hughes. 1989. Antibody-dependent cellular cytotoxicity of vascular endothelium: characterization and pathogenic associations in systemic sclerosis. *Clin. exp. Immunol.* 78:359-365.

147. Howe, S., E. Y. Eaker, J. E. Sallustio, C. Peebles, E. M. Tan, and R. C. Williams,Jr. 1994. Antimyenteric neuronal antibodies in scleroderma. *J. Clin. Invest.* 94:761-770.

148. Hsieh, T. -s. 1992. DNA topoisomerases. *Curr. Biol.* 4:396-400.

149. Huet, J., A. Sentenac, and P. Fromageot. 1982. Spot-immunodetection of conserved determinants in eukaryotic RNA polymerases. *J. Biol. Chem.* 257:2613-2618.

150. Huffstutter, J. E., F. A. DeLustro, and E. C. LeRoy. 1985. Cellular immunity to collagen and laminin in scleroderma. *Arthritis Rheum.* 28:775-780.

151. Hultman, P., S. Enestrom, K. M. Pollard, and E. M. Tan. 1989. Anti-fibrillarin autoantibodies in mercury-treated mice. *Clin. exp. Immunol.* 78:470-472.

152. Hultman, P., S. Enestrom, S. J. Turley, and K. M. Pollard. 1994. Selective induction of anti-fibrillarin autoantibodies by silver nitrate in mice. *Clin. exp. Immunol.* 96:285-291.

153. Imai, H., R. L. Ochs, K. Kiyosawa, S. Furuta, R. M. Nakamura, and E. M. Tan. 1992. Nucleolar antigens and autoantibodies in hepatocellular carcinoma and other malignancies. *Am. J. Pathol.* 140:859-870.

154. Incaprera, M., L. Rindi, A. Bazzichi, and C. Garzelli. 1998. Potential role of the Epstein-Barr virus in systemic lupus erthematosus autoimmunity. *Clin. Exp. Rheumatol.* 16:289-294.

155. Irani, A. -M. A., B. L. Gruber, L. D. Kaufman, M. B. Kahaleh, and L. B. Schwarz. 1992. Mast cell changes in scleroderma. Presence of MC_T cells in the skin and evidence of mast cell activation. *Arthritis Rheum.* 35:933-939.

156. Isenberg, D. A. and P. J. Maddison. 1987. Detection of antibodies to double stranded DNA and extractable nuclear antigen. *Assoc. Clin. Pathol. Broadsheet* 117:1-8.

157. Jaffee, B. D. and H. N. Claman. 1983. Chronic graft-versus-host disease (GVHD) as a model for scleroderma. *Cell immunol.* 77:1-12.

158. James, J. A., T. Gross, R. H. Scofield, and J. B. Harley. 1995. Immunoglobulin epitope spreading and autoimmune disease after peptide immunization: Sm B/B'-derived PPPGMRPP and PPPGIRGP induce spliceosome autoimmunity. *J. Exp. Med.* 181:453-461.

159. James, J. P., T. R. J. Stevens, N. D. Hall, P. J. Maddison, N. J. Goulding, A. Silman, S. Holligan, and C. Black. 1990. Factor VIII related antigen in connective tissue disease patients and relatives. *Br. J. Rheumatol.* 29:6-9.

160. Janwityanuchit, S., M. Vanichapunta, O. Verasertniyom, K. Totemchokchyakarn, and M. Vatanasuk. 1994. Antinucleolar antibodies and their disease association. *Asian Pacific J. Allergy Immunol.* 12:43-49.

161. Jarzabek-Chorzelska, M., M. Blaszczyk, Z. Kolacinska-Strasz, S. Jablonska, T. Chorzelski, and G. G. Maul. 1990. Are ACA and Scl 70 antibodies mutually exclusive? *Br. J. Dermatol.* 122:201-208.

162. Jelaska, A., M. Arakawa, G. Broketa, and J. H. Korn. 1996. Heterogeneity of collagen synthesis in normal and systemic sclerosis skin fibroblasts. Increased proportion of high collagen-producing cells in systemic sclerosis fibroblasts. *Arthritis Rheum.* 39:1338-1346.

163. Jodo, S., S. Kobayashi, N. Kayagaki, N. Ogura, Y. Feng, Y. Amasaki, A. Fujisaku, M. Azuma, H. Yagita, K. Okumura, and T. Koike. 1997. Serum levels of soluble Fas/APO-1 (CD95) and its molecular structure in patients with systemic lupus erythematosus (SLE) and other autoimmune diseases. *Clin. exp. Immunol.* 107:89-95.

164. Kahaleh, B. and M. Matucci-Cerinic. 1995. Raynaud's phenomenon and scleroderma. Dysregulated neuroendothelial control of vascular tone. *Arthritis Rheum.* 38:1-4.

165. Kahaleh, M. B. 1990. Vascular disease in scleroderma. Endothelial T lymphocyte-fibroblast interactions. *Rheum. Dis. Clin. N. Amer.* 16:53-73.

166. Kahaleh, M. B. 1991. Endothelin, an endothelial-dependent vasoconstrictor in scleroderma. Enhanced producton and profibrotic action. *Arthritis Rheum.* 34:978-983.

167. Kahaleh, M. B. 1995. Raynaud's phenomenon and the vascular disease in scleroderma. *Curr. Opin. Rheumatol.* 7:529-534.

168. Kallenberg, C. G. M. 1990. Early detection of connective tissue disease in patients with Raynaud's phenomenon. *Rheum. Dis. Clin. N. Amer.* 16:11-30.

169. Kallenberg, C. G. M. 1995. Overlapping syndromes, undifferentiated connective tissue disease, and other fibrosing conditions. *Curr. Opin. Rheumatol.* 7:568-573.

170. Kallenberg, C. G. M., G. W. Pastoor, A. A. Wouda, and T. H. The. 1982. Antinuclear antibodies in patients with Raynaud's phenomenon: clinical significance of anticentromere antibodies. *Ann. Rheum. Dis.* 41:382-387.

171. Kallenberg, C. G. M., A. A. Wouda, M. H. Hoet, and W. J. van Venrooij. 1988. Development of connective tissue disease in patients presenting with Raynaud's phenomenon: a six year follow up with emphasis on the predictive value of antinuclear antibodies as detected by immunoblotting. *Ann. Rheum. Dis.* 47:634-641.

172. Kanda, N., T. Tsuchida, and K. Tamaki. 1997. Testosterone suppresses anti-DNA antibody production in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Arthritis Rheum.* 40:1703-1711.

173. Kaufman, L. D. and L. B. Krupp. 1995. Eosinophilia-myalgia syndrome, toxic-oil syndrome, and diffuse fasciitis with eosinophilia. *Curr. Opin. Rheumatol.* 7:560-567.

174. Kawaguchi, Y., K. Suzuki, M. Hara, T. Hidaka, T. Ishizuka, M. Kawagoe, and H. Nakamura. 1994. Increased endothelin-1 production in fibroblasts derived from patients with systemic sclerosis. *Ann. Rheum. Dis.* 53:506-510.

175. Kiener, H., W. Graninger, K. Machold, M. Aringer, and W. B. Graninger. 1994. Increased levels of circulating intercellular adhesion molecule-1 in patients with systemic sclerosis. *Clin. Exp. Rheumatol.* 12:483-487.

176. Kikuchi, K., C. W. Hartl, E. A. Smith, E. C. LeRoy, and M. Trojanowska. 1992. Direct demonstration of transcriptional activation of collagen gene expression in systemic sclerosis fibroblasts: insensitivity to TGFβ1 stimulation. *Biochem. Biophysical Res. Commun.* 187:45-50.

177. Kipnis, R. J., J. Craft, and J. A. Hardin. 1990. The analysis of antinuclear and antinucleolar autoantibodies of scleroderma by radioimmunoprecipitation assays. *Arthritis Rheum.* 33:1431-1437.

178. Kondo, H., B. S. Rabin, and G. P. Rodnan. 1976. Cutaneous antigen-stimulating lymphokine production by lymphocytes of patients with progressive systemic sclerosis (scleroderma). *J. Clin. Invest.* 58:1388-1394.

179. Kosco-Vilbois, M. H., D. Gray, D. Scheidegger, and M. Julius. 1993. Fillicular dendritic cells help resting B cells to become effective antigen-presenting cells: induction of B7/BB1 and upregulation of major histocompatibility complex Class II molecules. *J. Exp. Med.* 178:2055-2066.

180. Kretzschmar, M., M. Meisterernst, and R. G. Roeder. 1993. Identification of human DNA topoisomerase 1 as a cofactor for activator-dependent transcription by RNA polymerase II. *Proc. Natl. Acad. Sci. USA* 90:11508-11512.

181. Kurts, C., F. R. Carbone, M. Barnden, E. Blanas, J. Allison, W. R. Heath, and J. F. A. P. Miller. 1997. $CD4^+$ T cell help impairs $CD8^+$ T cell deletion induced by cross-presentation of self-antigens and favours autoimmunity. *J. Exp. Med.* 186:2057-2062.

182. Kuwana, M., T. Fujii, T. Mimori, and J. Kaburaki. 1996. Enhancement of anti-DNA topoisomerase I autoantibody response after lung cancer in patients with systemic sclerosis. A report of two cases. *Arthritis Rheum.* 39:686-691.

183. Kuwana, M., J. Kaburaki, T. Mimori, T. Tojo, and M. Homma. 1993. Autoantibody reactive with three classes of RNA polymerases in sera from patients with systemic sclerosis. *J. Clin. Invest.* 91:1399-1404.

184. Kuwana, M., J. Kaburaki, Y. Okano, H. Inoko, and K. Tsuji. 1993. The HLA-DR and DQ genes control the autoimmune response to DNA topoisomerase I in systemic sclerosis (scleroderma). *J. Clin. Invest.* 92:1296-1301.

185. Kuwana, M., J. Kaburaki, Y. Okano, T. Tojo, and M. Homma. 1994. Clinical and prognostic associations based on serum antinuclear antibodies in Japanese patients with systemic sclerosis. *Arthritis Rheum.* 37:75-83.

186. Kuwana, M., T. A. Medsger,Jr, and T. M. Wright. 1995. T cell proliferative response induced by DNA topoisomerase I in patients with systemic sclerosis and healthy donors. *J. Clin. Invest.* 96:586-596.

187. Kuwana, M., T. A. Medsger,Jr, and T. M. Wright. 1995. T and B cell collaboration is essential for the autoantibody response to DNA topoisomerase I in systemic sclerosis. *J. Immunol.* 155:2703-2714.

188. Kuwana, M., Y. Okano, J. Kaburaki, and H. Inoko. 1995. HLA class II genes associated with anticentromere antibody in Japanese patients with systemic sclerosis (scleroderma). *Ann. Rheum. Dis.* 54:983-987.

189. Kuwana, M., Y. Okano, J. Kaburaki, T. Tojo, and T. A. Medsger,Jr. 1994. Racial differences in the distribution of systemic sclerosis-related serum antinuclear antibodies. *Arthritis Rheum.* 37:902-906.

190. Kwok, W. W., G. T. Nepom, and F. C. Raymond. 1995. HLA-DQ polymorphisms are highly selective for peptide binding interactions. *J. Immunol.* 155:2468-2476.

191. Laffon, A. and R. Gonzalez-Amaro. 1995. Cell adhesion molecules: an overview. *Br. J. Rheumatol.* 34:1101-1104.

192. Laybourn, P. J. and M. E. Dahmus. 1990. Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promotor and before initiation of transcription. *J. Biol. Chem.* 265:13165-13173.

193. LeFeber, W. P., D. A. Norris, S. R. Ryan, J. C. Huff, L. A. Lee, M. Kubo, S. T. Boyce, B. L. Kotzin, and W. L. Weston. 1984. Ultraviolet light induces binding of antibodies to selected nuclear antigens on cultured human keratinocytes. *J. Clin. Invest.* 74:1545-1551.

194. Lehmann, P. V., T. Forsthuber, A. Miller, and E. E. Sercarz. 1992. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 358:155-157.

195. LeRoy, E. C. 1989. The pathogenesis of systemic sclerosis. *Clin. Exp. Rheumatol.* 7;Suppl.3:135-137.

196. LeRoy, E. C. 1992. A brief overview of the pathogenesis of scleroderma (systemic sclerosis). *Ann. Rheum. Dis.* 51:286-288.

197. LeRoy, E. C. 1993. Pathogenesis of systemic sclerosis. In Arthritis and allied conditions. A textbook of rheumatology. D. J. McCarthy and W. J. Koopman, editors. Lea and Febiger, London. 1293-1299.

198. LeRoy, E. C., C. Black, R. Fleischmajer, S. Jablonska, T. Krieg, T. A. Medsger,Jr, N. Rowell, and F. Wollheim. 1988. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J. Rheumatol.* 15:202-205.

199. LeRoy, E. C., E. A. Smith, M. B. Kahaleh, M. Trojanowska, and R. M. Silver. 1989. A strategy for determining the pathogenesis of systemic sclerosis. Is transforming growth factor beta the answer? *Arthritis Rheum.* 32:817-825.

200. Lipham, W. J., T. M. Redmond, H. Takahashi, J. A. Berzofsky, B. Wiggert, G. J. Chader, and I. Gery. 1991. Recognition of peptides that are immunopathogenic but cryptic. Mechanisms that allow lymphocytes sensitized against cryptic peptides to initiate pathogenic autoimmune processes. *J. Immunol.* 146:3757-3762.

201. Lischwe, M. A., R. L. Ochs, R. Reddy, R. G. Cook, L. C. Yeoman, E. M. Tan, M. Reichlin, and H. Busch. 1985. Purification and partial characterization of a nucleolar scleroderma antigen (M_r = 34,000; pI, 8.5) rich in N^G,N^G-dimethylarginine. *J. Biol. Chem.* 260:14304-14310.

202. Livingston, J. Z., T. E. Scott, F. M. Wigley, G. J. Anhalt, W. B. Bias, R. H. McLean, and M. C. Hochberg. 1987. Systemic sclerosis (scleroderma): Clinical, genetic, and serological subsets. *J. Rheumatol.* 14:512-518.

203. Lopez-Hoyos, M., R. Carrio, R. Merino, L. Buelta, S. Izui, G. Nunez, and J. Merino. 1996. Constitutive expression of Bcl-2 in B cells causes a lethal form of lupuslike autoimmune disease after induction of neonatal tolerance to H-2^b alloantigens. *J. Exp. Med.* 183:2523-2531.

204. Lu, H., O. Flores, R. Weinmann, and D. Reinberg. 1991. The non-phosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. *Proc. Natl. Acad. Sci. USA* 88:10004-10008.

205. Maddison, P. J. 1993. Autoantibody profile. In Oxford textbook of rheumatology. Oxford University Press, Oxford. 389-396.

206. Maddison, P. J., R. P. Skinner, R. S. Pereira, C. M. Black, B. M. Ansell, M. I. V. Jayson, N. R. Rowell, and K. I. Welsh. 1986. Antinuclear antibodies in the relatives and spouses of patients with systemic sclerosis. *Ann. Rheum. Dis.* 45:793-799.

207. Maddison, P. J., C. Stephens, D. Briggs, K. I. Welsh, G. Harvey, J. Whyte, N. McHugh, C. M. Black, and The United Kingdom Systemic sclerosis Study Group. 1993. Connective tissue disease and autoantibodies in the kindreds of 63 patients with systemic sclerosis. *Medicine (Baltimore)* 72:103-112.

208. Maguerie, C., C. C. Bunn, J. Copier, R. M. Bernstein, J. M. Gilroy, C. M. Black, A. K. So, and M. J. Walport. 1992. The clinical and immunogenetic features of patients with autoantibodies to the nucleolar antigen Pm-Scl. *Medicine* 71:327-336.

209. Mamula, M. J. 1993. The inability to process a self-peptide allows autoreactive T cells to escape tolerance. *J. Exp. Med.* 177:567-571.

210. Mamula, M. J. 1995. Lupus autoimmunity: from peptides to particles. *Imm. Rev.* 144:301-315.

211. Mamula, M. J., S. Fatenejad, and J. Craft. 1994. B cells process and present lupus autoantigens that initiate autoimmune T cell responses. *J. Immunol.* 152:1453-1461.

212. Mamula, M. J. and C. A. Janeway,Jr. 1993. Do B cells drive the diversification of immune responses? *Immunol. Today* 14:151-152.

213. Mamula, M. J., R. -H. Lin, C. A. Janeway,Jr, and J. A. Hardin. 1992. Breaking T cell tolerance with foreign and self co-immunogens. A study of autoimmune B and T cell epitopes of cytochrome c. *J. Immunol.* 149:789-795.

214. Maquart, F. -X., P. Gillery, and B. Kalis. 1993. Environmental factors in scleroderma and cytokines: the link. *Eur. J. Dermatol.* 3:286-287.

215. Maricq, H. R. 1981. Widefield capillary microscopy. Technique and rating scale for abnormalities seen in scleroderma and related disorders. *Arthritis Rheum.* 24:1159-1165.

216. Marks, R. M., M. Czerniecki, B. S. Andrews, and R. Penny. 1988. The effects of scleroderma serum on human microvascular endothelial cells. Induction of antibody-dependent cellular cytotoxicity. *Arthritis Rheum.* 31:1524-1534.

217. Masi, A. T., S. L. Feigenbaum, R. T. Chatterton, and M. Cutolo. 1995. Integrated hormonal-immunological-vascular (H-I-V triad) systems interactions in the rheumatic diseases. *Clin. Exp. Rheumatol.* 13:203-216.

218. Masi, A. T., G. P. Rodnan, T. A. Medsger,Jr, R. D. Altman, W. A. D'Angelo, J. F. Fries, E. C. LeRoy, A. B. Kirsner, A. H. MacKenzie, D. J. McShane, A. R. Myers, and G. C. Sharp. 1980. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum.* 23:581-590.

219. Masson, M., V. Rolli, F. Dantzer, C. Trucco, V. Screiber, S. Fribourg, M. Molinete, A. Ruf, E. Alves Miranda, C. Niedergang, D. Hunting, B. Gowans, G. E. Schulz, J. M. de Murcia, and G. de Murcia. 1995. Poly(ADP-ribose) polymerase: structure-function relationship. *Biochimie* 77:456-461.

220. Matunis, E. L., M. J. Matunis, and G. Dreyfuss. 1993. Association of individual hnRNP proteins and snRNPs with nascent transcripts. *J. Cell Biol.* 121:219-228.

221. Maul, G. G., B. T. French, W. J. van Venrooij, and S. A. Jimenez. 1986. Topoisomerase I identified by scleroderma 70 antisera: Enrichment of topoisomerase I at the centromere in mouse mitotic cells before anaphase. *Proc. Natl. Acad. Sci. USA* 83:5145-5149.

222. Maul, G. G., S. A. Jimenez, E. Riggs, and D. Ziemnicka-Kotula. 1989. Determination of an epitope of the diffuse systemic sclerosis marker antigen DNA topoisomerase I: sequence similarity with retroviral p30^{gag} protein suggests a possible cause for autoimmunity in systemic sclerosis. *Proc. Natl. Acad. Sci. USA* 86:8492-8496.

223. McColl, G. J. and R. R. C. Buchanan. 1993. Familial CREST syndrome. *J. Rheumatol.* 21:754-756.

224. McFarland, H. F. 1996. Complexities in the treatment of autoimmune disease. *Science* 274:2037-2038.

225. McHugh, N. J., I. E. James, and P. J. Maddison. 1988. Differential isotype recognition of two centromere associated polypeptides by immunoblotting in connective tissue disease. *Clin. exp. Immunol.* 72:457-464.

226. McHugh, N. J., J. Whyte, C. Artlett, D. C. Briggs, C. O. Stephens, N. J. Olsen, N. G. Gusseva, P. J. Maddison, C. M. Black, and K. Welsh. 1994. Anti-centromere antibodies (ACA) in systemic sclerosis patients and their relatives: a serological and HLA study. *Clin. exp. Immunol.* 96:267-274.

227. McHugh, N. J., J. Whyte, G. Harvey, and U. F. Haustein. 1994. Anti-topoisomerase I antibodies in silica-associated systemic sclerosis. A model for autoimmunity. *Arthritis Rheum.* 37:1198-1205.

228. McNeilage, L. J., U. Youngchaiyud, and S. Whittingham. 1989. Racial differences in antinuclear antibody patterns and clinical manifestations of scleroderma. *Arthritis Rheum.* 32:54.

229. Medsger, T. A.,Jr 1993. Systemic sclerosis (scleroderma), localized forms of scleroderma, and calcinosis. In Arthritis and allied conditions. A textbook of rheumatology. D. J. McCarthy and W. J. Koopman, editors. Lea and Febiger, London. 1253-1292.

230. Merino, A., K. R. Madden, W. S. Lane, J. J. Champoux, and D. Reinberg. 1993. DNA topoisomerase I is involved in both repression and activation of transcription. *Nature* 365:227-232.

231. Miller, F. W., S. A. Twitty, T. Biswas, and P. H. Plotz. 1990. Origin and regulation of a disease-specific autoantibody response. Antigenic epitopes, spectrotype stability, and isotype restriction of anti-Jo-1 autoantibodies. *J. Clin. Invest.* 85:468-475.

232. Mimori, T., J. A. Hardin, and J. A. Steitz. 1986. Characterization of the DNA-binding protein antigen Ku recognized by autoantibodies from patients with rheumatic disorders. *J. Biol. Chem.* 261:2274-2278.

233. Mimori, T., M. Hinterberger, I. Pettersson, and J. A. Steitz. 1984. Autoantibodies to the U2 small nuclear ribonucleoprotein in a patient with scleroderma-polymyositis overlap syndrome. *J. Biol. Chem.* 259:560-565.

234. Mimori, T., Y. Ohosone, N. Hama, A. Suwa, M. Akizuki, M. Homma, A. J. Griffith, and J. A. Hardin. 1990. Isolation and characterization of cDNA encoding the 80-kDa subunit protein of the human autoantigen Ku (p70/p80) recognized by autoantibodies from patients with scleroderma-polymyositis overlap syndrome. *Proc. Natl. Acad. Sci. USA* 87:1777-1781.

235. Molta, C. T., M. A. Khan, C. J. Aponte, T. L. Reynolds, and S. S. Macintyre. 1989. Familial occurrence of systemic sclerosis, rheumatoid arthritis and other immunological disorders: report of two kindreds with study of HLA antigens and review of the literature. *Clin. Exp. Rheumatol.* 7:229-236.

236. Morse, J. H., M. Fotino, Y. Zhang, E. R. Flaster, C. L. Peebles, and H. Spiera. 1995. Position 26 of the first domain of the HLA-DQB1 allele in post-silicone implant scleroderma . *J. Rheumatol.* 22:1872-1875.

237. Mountz, J. D., C. K. Edwards,III, J. Cheng, P. Yang, Z. Wang, C. Liu, X. Su, H. Bluethmann, and T. Zhou. 1996. Autoimmunity due to defective NUR77, Fas, and TNF-RI apoptosis. In Mechanisms of lymphocyte activation and immune regulation VI. Gupta and Cohen, editors. Plenum Press, New York. 241-262.

238. Mountz, J. D., J. Wu, J. Cheng, and T. Zhou. 1994. Autoimmune disease: A problem of defective apoptosis. *Arthritis Rheum.* 37:1415-1420.

239. Mouritsen, S., A. S. Hansen, B. L. Petersen, and S. Buus. 1992. pH dependence of the interaction between immunogenic peptides and MHC Class II molecules. Evidence for an acidic intracellular compartment being the organelle of interaction. *J. Immunol.* 148:1438-1444.

240. Muller, S., J. -P. Briand, S. Barakat, J. Lagueux, G. G. Poirier, G. de Murcia, and D. A. Isenberg. 1994. Autoantibodies reacting with poly(ADP-ribose) and with a zinc-finger functional domain of poly(ADP-ribose) polymerase involved in the recognition of damaged DNA. *Clin. Immunol. Immunopathol.* 73:187-196.

241. Mund, D. J. and R. A. Greenwald. 1978. The CREST syndrome variant of scleroderma in a mother-daughter pair. *J. Rheumatol.* 5:307-310.

242. Negi, V. S., N. K. Tripathy, R. Misra, and S. Nityanand. 1998. Antiendothelial cell antibodies in scleroderma correlate with severe digital ischaemia and pulmonary arterial hypertension. *J. Rheumatol.* 25:461-465.

243. Nelson, J. L. 1996. Maternal-Fetal immunology and autoimmune disease. Is some autoimmune disease auto-alloimmune or allo-autoimmune. *Arthritis Rheum.* 39:191-194.

244. Nelson, J. L., D. E. Furst, S. Maloney, T. Gooley, P. C. Evans, A. Smith, M. A. Bean, C. Ober, and D. W. Bianchi. 1998. Microchimerism and HLA-compatible relationships of pregnancy in scleroderma. *The Lancet* 351:559-562.

245. Nepom, B. S. and G. T. Nepom. 1995. Polyglot and polymorphism. An HLA update. *Arthritis Rheum.* 12:1715-1751.

246. Nosseri, C., S. Coppola, and L. Ghibelli. 1994. Possible involvement of poly(ADP-ribosyl) polymerase in triggering stress-induced apoptosis. *Exp. Cell Res.* 212:367-373.

247. Nunez, G., R. Merino, D. Grillot, and M. Gonzalez-Garcia. 1994. Bcl-2 and Bcl-x: regulatory switches for lymphoid death and survival. *Immunol. Today* 15:582-588.

248. O'Brien, C. A. and S. L. Wolin. 1994. A possible role for the 60-kD Ro autoantigen in a discard pathway for defective 5S rRNA precursors. *Genes Dev.* 2891-2903.

249. Ochel, M., H. W. Vohr, C. Pfeiffer, and E. Gleichmann. 1991. IL-4 is required for the IgE and IgG1 increase and IgG1 autoantibody formation in mice treated with mercuric chloride. *J. Immunol.* 146:3006-3011.

250. Ochs, R. L. and R. I. Press. 1992. Centromere autoantigens are associated with the nucleolus. *Exp. Cell Res.* 200:339-350.

251. Oddis, C. V., Y. Okano, W. A. Rudert, M. Trucco, R. J. Duquesnoy, and T. A. Medsger,Jr. 1992. Serum autoantibody to the nucleolar antigen PM-Scl. Clinical and immunogenetic associations. *Arthritis Rheum.* 35:1211-1217.

252. Okano, Y. and T. A. Medsger,Jr. 1990. Autoantibody to Th ribonucleoprotein (nucleolar 7-2 RNA protein particle) in patients with systemic sclerosis. *Arthritis Rheum.* 33:1822-1828.

253. Okano, Y. and T. A. Medsger,Jr. 1991. Newly identified U4/U6 snRNP-binding proteins by serum autoantibodies from a patient with systemic sclerosis. *J. Immunol.* 146:535-542.

254. Okano, Y., V. D. Steen, and T. A. Medsger,Jr. 1992. Autoantibody to U3 nucleolar ribonucleoprotein (fibrillarin) in patients with systemic sclerosis. *Arthritis Rheum.* 35:95-100.

255. Okano, Y., V. D. Steen, and T. A. Medsger,Jr. 1993. Autoantibody reactive with RNA polymerase III in systemic sclerosis. *Ann. Intern. Med.* 119:1005-1013.

256. Oldstone, M. B. A. 1987. Molecular mimicry and autoimmune disease. *Cell* 50:819-820.

257. Ota, H., S. Kumagai, A. Morinobu, and K. Nakao. 1995. Enhanced production of transforming growth factor-beta (TGF-beta) during autologous mixed lymphocyte reaction of systemic sclerosis patients. *Clin. exp. Immunol.* 100:99-103.

258. Otsuki, T., H. Sakaguchi, A. Tmokuni, T. Aikoh, T. Matsuki, Y. Kawakami, and M. Kusaka. 1998. Soluble Fas mRNA is dominantly expressed in cases with silicosis. *Immunology* 94:258-262.

259. Ottenhoff, T. H. M. 1996. Selective modulation of T-cell responses in autoimmunity and cancer. *Immunol. Today* 17:54-55.

260. Pandey, J. P. and E. C. LeRoy. 1998. Human cytomegalovirus and the vasculopathies of autoimmune diseases (especially scleroderma), allograft rejection, and coronary restinosis. *Arthritis Rheum.* 41:10-15.

261. Parodi, A., P. Puiatti, and A. Rebora. 1991. Serological profiles as prognostic clues for progressive systemic scleroderma: The italian experience. *Dermatologica* 183:15-20.

262. Payne, J. M., P. J. Laybourn, and M. E. Dahmus. 1989. The transition of RNA polymerase II from initiation to elongation is associated with phosphorylation of the carboxyl-terminal domain of subunit IIa. *J. Biol. Chem.* 264:19621-19629.

263. Pearson, J. D. 1991. The endothelium: its role in scleroderma. *Ann. Rheum. Dis.* 50:866-871.

264. Pereira, S., C. Black, K. Welsh, B. Ansell, M. Jayson, P. Maddison, and N. Rowell. 1987. Autoantibodies and immunogenetics in 30 patients with systemic sclerosis and their families. *J. Rheumatol.* 14:760-765.

265. Perez, M. I. and S. R. Kohn. 1993. Systemic sclerosis. *J. Amer. Acad. Dermatol.* 28:525-547.

266. Pignone, A., C. Scaletti, M. Matucci-Cerinic, D. Vazquez-Abad, P. L. Meroni, N. Del Papa, F. Falcini, S. Generini, N. Rothfield, and M. Cagnoni. 1998. Anti-endothelial cell antibodies in systemic sclerosis: significant association with vascular involvement and alveolo-capillary impairment. *Clin. Exp. Rheumatol.* 16:527-532.

267. Pisetsky, D. S. Rheumatic disease etiology: immune-mediated inflammation. In . 13.1-13.6.

268. Playfair, J. H. L. 1992. Immunology at a glance. Blackwell Scientific Publications, Oxford.

269. Plunkett, M. L. and T. A. Springer. 1986. Purification and characterization of the lymphocyte function-associated-2 (LFA-2) molecule. *J. Immunol.* 136:4181-4187.

270. Pollard, K. M., D. K. Lee, C. A. Casiano, M. Bluthner, M. M. Johnston, and E. M. Tan. 1997. The autoimmunity-inducing xenobiotic mercury interacts with the autoantigen fibrillarin and modifies its molecular and antigenic properties. *J. Immunol.* 158:3521-3528.

271. Pollard, K. M., G. Reimer, and E. M. Tan. 1989. Autoantibodies in scleroderma. *Clin. Exp. Rheumatol.* 7/S-3:57-62.

272. Postlethwaite, A. E. 1990. Early immune events in scleroderma. *Rheum. Dis. Clin. N. Amer.* 16:125-139.

273. Query, C. C. and J. D. Keene. 1987. A human autoimmune protein associated with U1RNA contains a region of homology that is cross-reactive with retroviral p30^{gag} antigen. *Cell* 51:211-220.

274. Reddy, R., E. M. Tan, D. Henning, K. Nohga, and H. Busch. 1983. Detection of a nucleolar 7-2 ribonucleoprotein and a cytoplasmic 8-2 ribonucleoprotein with autoantibodies from patients with scleroderma. *J. Biol. Chem.* 258:1383-1386.

275. Reichlin, M. and F. C. Arnett,Jr. 1984. Multiplicity of antibodies in myositis sera. *Arthritis Rheum.* 27:1150-1156.

276. Reimer, G. 1990. Autoantibodies against nuclear, nucleolar, and mitochondrial antigens in systemic sclerosis (scleroderma). *Rheum. Dis. Clin. N. Amer.* 16:169-183.

277. Reimer, G., K. M. Rose, U. Scheer, and E. M. Tan. 1987. Autoantibody to RNA polymerase I in scleroderma sera. *J. Clin. Invest.* 79:65-72.

278. Reimer, G., V. D. Steen, C. A. Penning, T. A. Medsger,Jr, and E. M. Tan. 1988. Correlates between autoantibodies to nucleolar antigens and clinical features in patients with systemic sclerosis (scleroderma). *Arthritis Rheum.* 31:525-532.

279. Reveille, J. D. 1995. Molecular genetics of systemic sclerosis. *Curr. Opin. Rheumatol.* 7:522-528.

280. Reveille, J. D., E. Durban, R. Goldstein, R. Moreda, and F. C. Arnett. 1992. Racial differences in the frequencies of scleroderma-related autoantibodies. *Arthritis Rheum.* 35:216-218.

281. Reveille, J. D., E. Durban, M. J. MacLoed-St.Clair, R. Goldstein, R. Moreda, R. D. Altman, and F. C. Arnett. 1992. Association of amino acid sequences in the HLA-DQB1 first domain with the antitopoisomerase I autoantibody response in scleroderma (progressive systemic sclerosis). *J. Clin. Invest.* 90:973-980.

282. Reveille, J. D., D. Owerbach, R. Goldstein, R. Moreda, R. A. Isern, and F. C. Arnett. 1992. Association of polar amino acids at position 26 of the HLA-DQB1 first domain with the anticentromere autoantibody response in systemic sclerosis (scleroderma). *J. Clin. Invest.* 89:1208-1213.

283. Reynolds, P., T. P. Gordon, A. W. Purcell, D. C. Jackson, and J. McCluskey. 1996. Hierarchical self-tolerance to T cell determinants within the ubiquitous nuclear self-antigen La (SS-B) permits induction of systemic autoimmunity in normal mice. *J. Exp. Med.* 184:1857-1870.

284. Rhodes, G., H. Rumpold, P. Kurki, K. M. Patrick, D. A. Carson, and J. H. Vaughan. 1987. Autoantibodies in infectious mononucleosis have specificity for the glycine-alanine repeating region of the Epstein-Barr virus nuclear antigen. *J. Exp. Med.* 165:1026-1040.

285. Riente, L., B. Marchini, M. P. Dolcher, A. Puccetti, S. Bombardieri, and P. Migliorini. 1995. Anti-collagen antibodies in systemic sclerosis and in primary Raynaud's phenomenon. *Clin. exp. Immunol.* 102:354-359.

286. Rodriguez-Sanchez, J. L., C. Gelpi, C. Juarez, and J. A. Hardin. 1987. Anti-Nor 90. A new autoantibody in scleroderma that recognizes a 90-kDa component of the nucleolus-organizing region of chromatin. *J. Immunol.* 139:2579-2584.

287. Rosen, A., L. Casciola-Rosen, and J. Ahearn. 1995. Novel packages of viral and self-antigens are generated during apoptosis. *J. Exp. Med.* 181:1557-1561.

288. Rosen, A., L. Casciola-Rosen, and F. Wigley. 1997. Role of metal-catalyzed oxidation reactions in the early pathogenesis of scleroderma. *Curr. Opin. Rheumatol.* 9:538-543.

289. Rothfield, N. F. and G. P. Rodnan. 1968. Serum antinuclear antibodies in progressive systemic sclerosis (scleroderma). *Arthritis Rheum.* 11:607-617.

290. Roumm, A. D. and T. A. Medsger,Jr. 1985. Cancer and systemic sclerosis. An epidemiological study. *Arthritis Rheum.* 28:1336-1340.

291. Rudnicka, L., J. Varga, A. M. Christiano, R. V. Iozzo, S. A. Jimenez, and J. Uitto. 1994. Elevated expression of type VII collagen in the skin of patients with systemic sclerosis. *J. Clin. Invest.* 93:1709-1715.

292. Ruffatti, A., L. Artifoni, S. Glorioso, A. Calligaro, A. Doria, P. Gambari, and S. Todesco. 1985. Prevalence of anticentromere antibody in blood relatives of anticentromere posiyive patients. *J. Rheumatol.* 12:940-943.

293. Rustin, M. H. A., H. A. Bull, V. Ziegler, J. Mehlhorn, U. -F. Haustein, P. J. Maddison, J. James, and P. M. Dowd. 1990. Silica-associated systemic sclerosis is clinically, serologically and immunologically indistinguishable from idiopathic systemic sclerosis. *Br. J. Dermatol.* 123:725-734.

294. Sacks, D. G., Y. Okano, V. D. Steen, E. Curtiss, L. S. Shapiro, and T. A. Medsger,Jr. 1996. Isolated pulmonary hypertension in systemic sclerosis with diffuse cutaneous involvement: association with serum anti-U3RNP antibody. *J. Rheumatol.* 23:639-642.

295. Sanchez-Roman, J., I. Wichmann, J. Salaberri, J. M. Varela, and A. Nunez-Roldan. 1993. Multiple clinical and biological autoimmune manifestations in 50 workers after occupational exposure to silica. *Ann. Rheum. Dis.* 52:534-538.

296. Sato, S., H. Ihn, Y. Soma, M. Shimozuma, T. Shishiba, and K. Takehara. A case of systemic sclerosis with anticentromere, antitopoisomerase I, and anti-U1RNP antibodies. *Unknown* 1961-1963.

297. Satoh, M., A. K. Ajmani, T. Ogasawara, J. J. Langdon, M. Hirakata, and J. Wang. 1994. Autoantibodies to RNA polymerase II are common in systemic lupus erythematosus and overlap syndrome. Specific recognition of the phosphorylated (IIO) form by a subset of human sera. *J. Clin. Invest.* 94:1981-1989.

298. Satoh, M., M. Kuwana, T. Ogasawara, A. K. Ajmani, J. J. Langdon, D. Kimpel, and J. Wang. 1994. Association of autoantibodies to topoisomerase I and the phosphorylated (IIO) form of RNA polymerase in Japanese scleroderma patients. *Arthritis Rheum.* 37 (Suppl.):(Abstr.)

299. Satoh, M., M. Kuwana, T. Ogasawara, A. K. Ajmani, J. J. Langdon, D. Kimpel, J. Wang, and W. H. Reeves. 1994. Association of autoantibodies to topoisomerase I and the phosphorylated (IIO) form of RNA polymerase II in Japanese scleroderma patients. *J. Immunol.* 153:5838-5848.

300. Satoh, M., J. J. Langdon, and C. H. Chou. 1994. Characterization of the Su antigen, a macromolecular complex of 100/102 and 200 kDa proteins recognized by autoantibodies in systemic rheumatic diseases. *Clin. Immunol. Immunopathol.* 73:132-141.

301. Schiffenbauer, J. and B. D. Schwartz. 1987. The HLA complex and its relationship to rheumatic diseases. *Rheum. Dis. Clin. N. Amer.* 13:463-485.

302. Schreiber, V., D. Hunting, C. Trucco, B. Gowans, D. Grunwald, G. de Murcia, and J. M. de Murcia. 1995. A dominant-negative mutant of human poly(ADP-ribose) polymerase affects cell recovery, apoptosis, and sister chromatid exchange following DNA damage. *Proc. Natl. Acad. Sci. USA* 92:4753-4757.

303. Scofield, R. H., W. E. Henry, B. T. Kurien, J. A. James, and J. B. Harley. 1996. Immunization with short peptides from the sequence of the systemic lupus erythematosus-associated 60-kDa Ro autoantigen results in anti-Ro ribonucleoprotein autoimmunity. *J. Immunol.* 156:4059-4066.

304. Seibold, J. R. Systemic Sclerosis. In Rheumatology, J.H. Clippel and P.A.Dieppe editors. Mosby International, London 8.1-8.12.

305. Seibold, J. R. and J. N. Harris. 1985. Plasma b-thromboglobulin in the differential diagnosis of Raynaud's phenomenon. *J. Rheumatol.* 12:99-103.

306. Sels, F., R. Westhovans, M. -P. Emonds, E. Vandermeulen, and J. Dequeker. 1997. HLA typing in a large family with multiple cases of different autoimmune diseases. *J. Rheumatol.* 24:856-859.

307. Senaldi, G., S. Lupoli, D. Vergani, and C. M. Black. 1989. Activation of the complement system in systemic sclerosis. Relationship to clinical severity. *Arthritis Rheum.* 32:1262-1267.

308. Sentenac, A. 1985. Eukaryotic RNA polymerases. *CRC Crit. Rev. Biochem.* 18:31-91.

309. Shall, S. 1996. Lupus and poly(ADP-ribose). *Lupus* 5:1-3.

310. Sheldon, W. B., D. P. Lurie, H. R. Maricq, M. B. Kahaleh, F. A. DeLustro, A. Gibofsky, and E. C. LeRoy. 1981. Three siblings with scleroderma (systemic sclerosis) and two with Raynaud's phenomenon from a single kindred. *Arthritis Rheum.* 24:668-676.

311. Shero, J. H., B. Bordwell, N. F. Rothfield, and W. C. Earnshaw. 1986. High titres of autoantibodies to topoisomerase I (Scl-70) in sera from scleroderma patients. *Science* 231:737-740.

312. Shores, E. W., R. A. Eisenberg, and P. L. Cohen. 1988. T-B collaboration in the in vitro anti-Sm autoantibody response of MRL/Mp-lpr/lpr mice. *J. Immunol.* 140:2977-2982.

313. Silman, A. J. and S. Jones. 1992. What is the contribution of occupational environmental factors to the occurrence of scleroderma in men? *Ann. Rheum. Dis.* 51:1322-1324.

314. Silman, A. J. and J. Newman. 1994. Genetic and environmental factors in scleroderma. *Curr. Opin. Rheumatol.* 6:607-611.

315. Silver, R. M. and S. Miller. 1990. Lung involvement in systemic sclerosis. *Rheum. Dis. Clin. N. Amer.* 16:199-216.

316. Simeon, C. P., A. Castro-Guardiola, V. Foaollosa, L. L. Armadans, C. Clemente, R. Solans, C. Perez-Bocanegra, J. Lima, and M. Vilardel. 1996. Systemic sclerosis in men: clinical and immunological differences. *Br. J. Rheumatol.* 35:910-911.

317. Singh, R. R., B. H. Hahn, and E. E. Sercarz. 1996. Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune deviation: Implications for antibody *vs.* T cell-mediated autoimmunity. *J. Exp. Med.* 183:1613-1621.

318. Smith, E. A. and E. C. LeRoy. 1998. Systemic sclerosis: Etiology and pathogenesis. In Rheumatology. J. H. Klippel and P. A. Dieppe, editors. Mosby International, London. 10.1-10.10.

319. Smolen, J. S. and G. Steiner. 1998. Mixed connective tissue disease. To be or not to be? *Arthritis Rheum.* 41:768-777.

320. Sollberg, S. and T. Krieg. 1996. New aspects in scleroderma research. *Int. Arch. Allergy Immunol.* 111:330-336.

321. Sollberg, S., J. Peltonen, J. Uitto, and S. A. Jimenez. 1992. Elevated expression of beta-1 and beta-2 integrins, intercellular adhesion molecule 1, endothelial leukocyte adhesion molecule i in the skin of patients with systemic sclerosis of recent onset. *Arthritis Rheum.* 35:290-298.

322. Southwood, T. R., P. J. Roberts-Thomson, M. J. Ahern, K. Shepherd, R. McEvoy, J. B. Ziegler, and J. Edmonds. 1990. Autoantibodies in patients with juvenile chronic arthritis and their immediate family relatives. *Ann. Rheum. Dis.* 49:968-972.

323. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature* 346:425-434.

324. Springer, T. A. 1993. Immunoaffinity chromatography. In Current protocols in molecular biology. Current Protocols, editor. Current Protocols, 10.11.1-10.11.7.

325. Steen, V. D. 1998. Systemic sclerosis: Practical problems. In Rheumatology. J. H. Klippel and P. A. Dieppe, editors. Mosby International, London. 12.1-12.12.

326. Steen, V. D. and T. A. Medsger,Jr. 1990. Epidemiology and natural history of systemic sclerosis. *Rheum. Dis. Clin. N. Amer.* 16:1-10.

327. Steen, V. D., D. L. Powell, and T. A. Medsger,Jr. 1988. Clinical correlations and prognosis based on serum autoantibodies in patients with systemic sclerosis. *Arthritis Rheum.* 31:196-203.

328. Steen, V. D., G. L. Ziegler, G. P. Rodnan, and T. A. Medsger,Jr. 1984. Clinical and laboratory associations of anticentromere antibody in patients with progressive systemic sclerosis. *Arthritis Rheum.* 27:125-131.

329. Stefano, J. E. 1984. Purified lupus antigen La recognizes an oligouridylate stretch common to the 3' termini of RNA polymerase III transcripts. *Cell* 36:145-154.

330. Steinman, L. 1995. Escape from "horror autotoxicus": pathogenesis and treatment of autoimmune disease. *Cell* 80:7-10.

331. Stephens, C. O., D. C. Briggs, J. Whyte, C. M. Artlett, A. B. Scherbakov, N. Olsen, N. G. Gusseva, N. J. McHugh, P. J. Maddison, K. I. Welsch, and C. M. Black. 1994. Familial scleroderma-evidence for environmental versus genetic trigger. *Br. J. Rheumatol.* 33:1131-1135.

332. Stewart, J. J. 1998. The female inactivation mosaic in systemic lupus erythematosus. *Immunol. Today* 19:352-357.

333. Stone, O. J. 1991. Autoimmunity as a secondary phenomenon in scleroderma (and so-called human adjuvant disease). *Med. Hypoth.* 34:127-130.

334. Strosberg, J. M., B. Peck, and E. D. Harris,Jr. 1977. Scleroderma with intestinal involvement: fatal in two of a kindred. *J. Rheumatol.* 4:46-52.

335. Suwa, A., M. Hirakata, Y. Takeda, Y. Okano, T. Mimori, S. Inada, F. Watanabe, H. Teraoka, W. S. Dynan, and J. A. Hardin. 1996. Autoantibodies to DNA-dependent protein kinase. Probes for the catalytic subunit. *J. Clin. Invest.* 97:1417-1421.

336. Takehara, K., Y. Moroi, and Y. Ishibashi. 1985. Antinuclear antibodies in the relatives of patients with systemic sclerosis. *Br. J. Dermatol.* 112:23-33.

337. Takemura, H., H. Suzuki, K. Yoshizaki, A. Ogata, T. Yuhara, T. Akama, K. Yamane, and H. Kashiwagi. 1992. Anti-interleukin-6 autoantibodies in rheumatic disease. *Arthritis Rheum.* 35:940-943.

338. Takeuchi, F., K. Nakano, H. Yamada, G. H. Hong, H. Nabeta, A. Yoshida, K. Matsuta, M. Bannai, K. Tokunaga, and K. Ito. 1994. Association of HLA-DR with progressive systemic sclerosis in Japanese. *J. Rheumatol.* 21:857-863.

339. Tan, E. M. 1989. Antinuclear antibodies: Diagnostic markers for autoimmune diseases and probes for cell biology. *Adv. Immunol.* 44:93-147.

340. Tan, E. M. 1991. Autoantibodies in pathology and cell biology. *Cell* 67:841-842.

341. Tan, E. M. 1994. Autoimmunity and apoptosis. *J. Exp. Med.* 179:1083-1086.

342. Tan, E. M. and H. G. Kunkel. 1966. Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. *J. Immunol.* 96:464-471.

343. Tan, E. M., G. P. Rodnan, I. Garcia, Y. Moroi, M. J. Fritzler, and C. Peebles. 1980. Diversity of antinuclear antibodies in progressive systemic sclerosis. Anticentromere antibody and its relationship to CREST syndrome. *Arthritis Rheum.* 23:617-625.

344. Targoff, I. N. and M. Reichlin. 1985. Nucleolar localization of the PM-Scl antigen. *Arthritis Rheum.* 28:226-230.

345. Tomokuni, A., T. Aikoh, T. Matsuki, Y. Isozaki, T. Otsuki, S. Kita, H. Ueki, M. Kusaka, T. Kishimoto, and A. Ueki. 1997. Elevated soluble Fas/APO-1 (CD95) levels in silicosis patients without clinical symptoms of autoimmune diseases or malignant tumours. *Clin. exp. Immunol.* 110:303-309.

346. Usheva, A., E. Maldonado, A. Goldring, H. Lu, C. Houbavi, D. Reinberg, and Y. Aloni. 1992. Specific interaction between the non-phosphorylated form of RNA polymerase II and the TATA-binding protein. *Cell* 69:871-881.

347. Utz, P. J., M. Hottelet, P. H. Schur, and P. Anderson. 1997. Proteins phosphorylated during stress-induced apoptosis are common targets for autoantibody production in patients with systemic lupus erythematosus. *J. Exp. Med.* 185:843-854.

348. Utz, U., W. E. Biddison, H. F. McFarland, D. E. McFarlin, M. Flerlage, and R. Martin. 1993. Skewed T-cell receptor repertoire in genetically identical twins correlates with multiple sclerosis. *Nature* 364:243-247.

349. van Venrooij, W. J., S. O. Stapel, H. Houben, W. J. Habets, C. G. M. Kallenberg, E. Penner, and L. B. van de Putte. 1985. Scl-86, a marker antigen for diffuse scleroderma. *J. Clin. Invest.* 75:1053-1060.

350. Vazquez-Abad, D. and N. F. Rothfield. 1996. Sensitivity and specificity of anti-Jo-1 antibodies in autoimmune diseases with myositis. *Arthritis Rheum.* 39:292-296.

351. Vidard, L., K. L. Rock, and B. Benacerraf. 1992. Heterogeneity in antigen processing by different types of antigen-presenting cells. Effect of cell culture on antigen processing ability. *J. Immunol.* 149:1905-1911.

352. von Boehmer, H. and P. Kisielow. 1991. How the immune system learns about self. *Sci. Amer.* 10/91:50-59.

353. von Muhlen, C. A. and E. M. Tan. 1995. Autoantibodies in the diagnosis of systemic rheumatic disease. *Sem. Arth. Rheum.* 24:323-358.

354. Wang, J. C. 1985. DNA topoisomerases. *Ann. Rev. Biochem.* 54:665-697.

355. Wang, Z. -Q., B. Auer, L. Stingl, H. Berghammer, D. Haidacher, M. Schweiger, and E. F. Wagner. 1995. Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. *Genes Dev.* 9:509-520.

356. Weeks, J. R., S. E. Hardin, J. Shen, J. M. Lee, and A. L. Greenleaf. 1993. Locus-specific variation in phosphorylation state of RNA polymerase II in vivo: correlations with gene activity and transcript processing. *Genes Dev.* 7:2329-2344.

357. Weiner, E. S., W. C. Earnshaw, J. -L. Senecal, B. Bordwell, P. Johnson, and N. F. Rothfield. 1988. Clinical associations of anticentromere antibodies and antibodies to topoisomerase I. A study of 355 patients. *Arthritis Rheum.* 31:378-385.

358. Weiner, E. S., S. Hildebrandt, J. -L. Senecal, L. Daniels, S. Noell, F. Joyal, A. Roussin, W. Earnshaw, and N. F. Rothfield. 1991. Prognostic significance of anticentromere antibodies and anti-topoisomerase I antibodies in Raynaud's disease. A prospective study. *Arthritis Rheum.* 34:68-77.

359. Welsh, K. 1998. Scleroderma: chimerism, the blind man, and the scientist. *The Lancet* 351:540-541.

360. Wesierska-Gadek, J., E. Penner, E. Hitchman, P. Kier, and G. Sauermann. 1992. Nucleolar proteins B23 and C23 as target antigens in chronic graft-versus-host disease. *Blood* 79:1081-1086.

361. Whyte, J., C. Artlett, G. Harvey, C. Stephens, K. Welsh, C. Black, P. J. Maddison, N. J. McHugh, and The United Kingdom Systemic sclerosis Study Group. 1994. HLA-DQB1 associations with anti-topoisomerase-1 antibodies in patients with systemic sclerosis and their first degree relatives. *J. Autoimmunity* 7:509-520.

362. Wigley, F. M. 1994. Clinical aspects of systemic and localized scleroderma. *Curr. Opin. Rheumatol.* 6:628-636.

363. Wong, O. 1996. A critical assessment of the relationship between silicone breast implants and connective tissue diseases. *Regulat. Toxicol. Pharmacol.* 23:74-85.

364. Woychik, N. A., S. Laiao, P. A. Kolodziej, and R. A. Young. 1990. Subunits shared by eukaryotic nuclear RNA polymerases. *Genes Dev.* 4:313-323.

365. Wraith, D. C., H. O. McDevitt, L. Steinman, and H. Acha-Orbea. 1989. T cell recognition as the target for immune intervention in autoimmune disease. *Cell* 57:709-715.

366. Xiaozhou, L., L. J. McNeilage, and S. Whittingham. 1989. Autoantibodies to the major nucleolar phosphoprotein B23 define a novel subset of patients with anticardiolipin antibodies. *Arthritis Rheum.* 32:1165-1169.

367. X, 1998. Immunoaffinity purification. In Antibodies. 511-552.

368. Yamanaka, H., E. H. Willis, C. A. Penning, C. L. Peebles, E. M. Tan, and D. A. Carson. Characterization of the human autoantibody response to poly(ADP-ribose) polymerase. *Unknown* 139-144.

369. Yamanaka, H., E. H. Willis, C. A. Penning, C. L. Peebles, E. M. Tan, and D. A. Carson. 1987. Human autoantibodies to poly(adenosine diphosphate-ribose) polymerase. *J. Clin. Invest.* 80:900-904.

370. Yamane, K., H. Kashiwagi, N. Suzuki, T. Miyauchi, M. Yanagisawa, K. Goto, and T. Masaki. 1991. Elevated plasma levels of endothelin-1 in systemic sclerosis. *Arthritis Rheum.* 34:243-244.

371. Yamanishi, Y., H. Maeda, S. Katayama, S. Ishioka, and M. Yamakido. 1996. Scleroderma-polymyositis overlap syndrome associated with anti-Ku antibody and rimmed vacuole formation. *J. Rheumatol.* 23:1991-1994.

372. Yates, F. 1934. *Journal of the royal statistical society* Suppl.1:217.

373. Young, R. A. 1991. RNA polymerase II. *Ann. Rev. Biochem.* 60:689-715.

374. Zell, R. 1989. Retroviruses turn up in more autoimmune diseases. *New Sci.* 12/08:27.

375. Zhang, J. and J. L. Corden. 1991. Phosphorylation causes a conformational change in the carboxyl-terminal domain of the mouse RNA polymerase II largest subunit. *J. Biol. Chem.* 266:2297-2302.

376. Zhang, J. and J. L. Corden. 1991. Identification of phosphorylation sites in the repetitive carboxyl-terminal domain of the mouse RNA polymerase II largest subunit. *J. Biol. Chem.* 266:2290-2296.